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Force and Contraction Velocity of the Middle Ear Muscles in the Cat and the Rabbit

By

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Abstract

Teig, E. *Force and contraction velocity of the middle ear muscles in the cat and the rabbit.* Acta physiol. scand. 1972. *84.* 1—10.

Contractions of the stapedius and the tensor tympani muscles in cats and rabbits have been elicited at controlled length by electric stimulation of their motor nerves at varying stimulus frequencies. Both muscles were stronger than found in previous investigations. The tetanic tension of the stapedius was an average of 13.9 g (cat) and 15.4 g (rabbit). The tetanic tension of the tensor tympani averaged 54.3 g (cat) and 32.3 g (rabbit). By comparing the data from cats with the findings of Wever and Bray (1937, 1942) each muscle developed sufficient tension to cause a reduction in the sound transmission of the middle ear of more than 20 dB. With a twitch contraction time between 20 and 30 ms both muscles must be classified as fast muscles. However, different degrees of submaximal stimulation gave evidence for an additional slow twitch component in the tensor tympani muscle.

Contraction of the two muscles of the middle ear, the stapedius and the tensor tympani muscles, causes a change in the transmission of sound through the middle ear. One important function attributed to these muscles is protection of the inner ear against high intensity sound pressure. However, the capacity of the muscles to reduce the sound pressure and the mechanism by which they exert this function is still uncertain.

Reflex contraction of the muscles reduces the cochlear microphonic potentials equal to a reduction in sound transmission of 18—22 dB (Wever, Vernon and Lawrence 1955, Galambos and Rupert 1958, Carmel and Starr 1963). However, by applying loads to the muscle tendons to match the known maximal contraction force of the middle ear muscles, the reductions of the microphonic potentials corresponded to a decrease of 7—10 dB only (Wever and Bray 1937, 1942, Wever *et al.* 1955). This discrepancy has led to the theory that the middle ear muscles have a special structural arrangement whereby the active fibres are not adding their tensions in

any simple way, but to a large extent are working in opposition to one another" (Wever *et al* 1955) An alternative theory is that the middle ear muscles are arranged structurally as other muscles, and that they act in a simple way by exerting tension through their tendons This theory requires that the maximal contraction force of the muscles is *greater than previously recorded* *This possibility remains open* since the important parameters of stimulus frequency and optimal resting length have not been varied systematically in previous investigations

The object of the present investigation was to test these hypotheses by measuring the maximum strength of the middle ear muscles at controlled stimulus rates and at various muscle lengths When correlated with the data of Wever and Bray (1937, 1942) the muscular tensions were found to be strong enough to explain the observed reduction in sound transmission by maintaining that the muscles exert a simple pull on the ossicular chain through the muscle tendons

In the rabbit, Wersall (1958) noted that the contraction time of the tensor tympani was about twice that of the stapedius muscle

Since a longer contraction time in a muscle usually is associated with great endurance and static functions, this interesting observation might reflect a possible functional differentiation of these muscles A second objective of this investigation was, therefore to study the difference between the two middle ear muscles The results show that the difference between the contraction velocity of these muscles is relatively small and that they both, when compared to other skeletal muscles, must be classified as fast muscles However, evidence was found for the existence of slower contracting elements in the tensor tympani in contrast to the stapedius which seemed to be a homogenously fast muscle

Methods

Adult rabbits (2 7 4 8 kg) and adult cats (2 2 4 5 kg) were used The rabbits were anesthetized with 10 mg chloralose and 750 mg urethane per kg b w injected i v A level of anesthesia at which withdrawal of the leg to pinching was just abolished was later maintained by injections of urethane alone usually 750 1000 mg every 2—4 h The cats were anesthetized with sodium pentobarbital (30 mg per kg b w injected i p), and the anesthesia was later maintained by i v injection of 10 20 mg every 2—4 h

The animals were tracheotomized and fixed securely in a head holder A retroauricular incision through the skin and the platysma exposed the sternomastoid and the clavotrapezius muscles which were cut at their attachments to the skull The facial nerve was cut where it emerged from the stylomastoid foramen The ear canal was cut through its cartilaginous part and pulled forward In cats this gave good access to the mastoid bulla In order to obtain good access to the mastoid bulla in rabbits the masseter muscle was dissected free and the mandibular ramus resected In both species subsequent removal of the tympanic membrane and the lateral part of the mastoid bulla gave wide exposure of the middle ear The incus and parts of the malleus were removed Where it passes along the Eustachian tube the motor nerve to the tensor tympani (Blevins 1963) was cut by a deep section through both the nerve and the cartilage of the tube The facial nerve from which the stapedius nerve branches off was cut where it passes in a groove on the medial wall of the middle ear

Contractions of the tensor tympani were recorded by a transducer attached to its tendon by a *metal pin* clamped around the remnant of the malleus assuring no distortion of the normal direction of pull Contractions of the stapedius muscle were recorded with the transducer connected to the tendon by a fine metal wire which was tied around the lenticular process of the stapes The stapes crurae were then cut The field of operation was then filled with liquid paraffin at a temperature of 39° C The temperature of the paraffin pool surrounding the

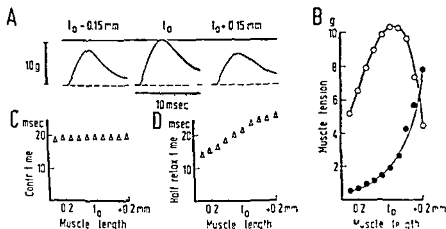


Fig 1 M stapedius rabbit A Maximal twitches obtained at optimal length (middle record) and after reducing and increasing this length by 0 15 mm (left and right record respectively) B Length tension diagram The maximal twitch tensions obtained at various muscular lengths are represented by open circles the corresponding resting tensions by filled circles C shows the contraction time and D the half relaxation time of twitches obtained at the indicated muscular lengths

muscles was measured by a thermocouple By frequent change of the paraffin the temperature was kept between 37 5 and 39° C. The body temperature was measured by a rectal thermometer and was kept within the same limits by controlled heating of the trunk region with an infrared lamp

The nerves to both muscles were stimulated supramaximally by unipolar silver electrodes (tip diameter 0 5 mm) applying square pulses of 0 2 ms duration In the case of the stapedius the cathode was placed on the distal stump of the severed facial nerve in the middle ear In the case of the tensor tympani the cathode was placed above the path of the nerve near the base of the muscle In spite of the proximity to the muscle the latter was not stimulated directly since the nerve had a lower threshold than the muscle The anode was placed on the neck musculature which was exposed during the operation

Isometric contractions of the muscles were recorded by an electromechanical transducer (RCA 5734) carried

sducer had a natural frequency
plied The output of the trans-
lied to the tip of the transducer
) to ?00 mV /g varying from one
played on an oscilloscope and
photographed

The transducer was linear for loads up to 20 g but higher loads gave a slightly curved calibration curve Measurements above 20 g were therefore read off a calibration graph

Results

The twitch tension and characteristic time relations of the contraction of a striated muscle are dependent on its initial length (Buller Eccles and Eccles 1960 Close 1964) For the middle ear muscles this initial length was found to be particularly critical The middle record of Fig 1 A shows a maximal twitch contraction of the

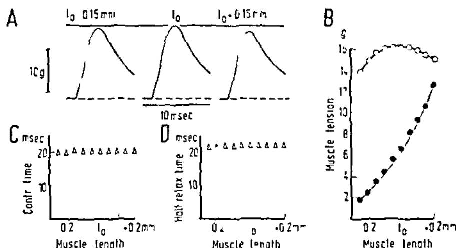


Fig 2 M tensor tympani rabbit Recording conditions and sequence as in Fig 1 A Maximal twitches obtained at various lengths B Length tension diagram C Contraction time and D half relaxation time

stapedius obtained at the optimal length The left and right hand record show twitches obtained after reducing and increasing the initial length by 0 15 mm respectively In the length tension diagram (Fig 1 B) the maximal twitch tensions obtained at various muscular lengths are represented by the open circles The corresponding resting tensions are given by the filled circles The diagram shows how a change in the initial length by only 0 2 mm reduced the twitches of the stapedius muscle to less than half the optimal size In contrast an increase of the muscle length gave only a small increase in the contraction time (Fig 1 C) while the half relaxation time increased considerably (Fig 1 D) Fig 2 contains the corresponding information for the tensor tympani muscle An increase or decrease of the resting length of 0 15 mm (Fig 2 A) gave a smaller change in the size and shape of the twitch than occurred in the stapedius muscle The length tension diagram (Fig 2 B) shows that a change in the resting length of 0 20 mm from the optimal length reduced the size of the twitch with about 10 per cent only The contraction time and the half relaxation time were hardly affected by such changes in the muscle length (Fig 2 C, D)

TABLE I Contraction properties of the [illegible] in the cat and rabbit

	Contraction time	Half relaxation time (ms)
m stapedius (cat)	21 [illegible] S D 0 [illegible]	25 4 S D 2 05
m tensor tympani cat	29 [illegible] S D 0 [illegible]	33 9 ± S D 1 28
m stapedius (rabbit)	22 0 S D 0 [illegible]	26 7 S D 1 87
m tensor tympani (rabbit	22 8 S D 0 [illegible]	27 1 S D 1 51

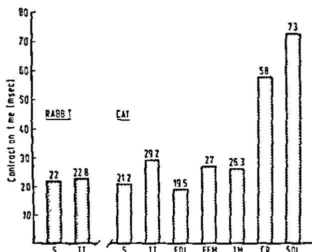


Fig 3 Graphic representation of the contraction time of the middle ear muscles of rabbit and cat and of various fast and slow twitch muscles of the cat S — m stapedius TT — m. tensor tympani EDL — m extensor digitorum longus (Gordon and Phillips 1953) FFM — pooled values of 6 fast flexor muscles of the leg (Buller Eccles and Eccles 1960) IM — intercostal muscles (Andersen and Sears 1964) CR — m crureus Sol — m soleus (Gordon and Phillips 1953)

In each experiment the optimal length was determined before the data for muscle twitches and tetani were obtained In different experiments the resting tension at the optimal length ranged between 1 5 and 2 5 g in the stapedius and from 2 to 6 g in the tensor tympani muscle The more detailed data on the contraction of the two muscles in both species are given in Table I The mean twitch contraction time of the stapedius muscle was 21 2 ms in the cat and 22 0 ms in the rabbit The corresponding values for the tensor tympani muscle was 29 2 ms in the cat and 22 8 ms in the rabbit

In Fig 3 are the contraction times of the middle ear muscles (S TT) correlated with the corresponding values for certain other muscles of the body found by other authors The contraction time of the two middle ear muscles is within the range of fast muscles

During the investigation it was noted that the last part of the relaxation phase of the tensor tympani was prolonged in both species This suggested that this muscle contained a slow component in addition to the fast one which contributed most of the twitch tension In order to test this hypothesis experiments with different degrees of submaximal stimulation were used Slow motor units are normally supplied by smaller alpha motor fibres than fast ones (Eccles Eccles and Lundberg 1958) Since

Twitch tension (g)	Tetanus tension (g)	Tetanus twitch ratio	Number of observations
3 70±S D 0 30	13 90±S D 1 84	3 9 S D 0 48	6
13 58±S D 1 29	54 25±S D 4 05	4 1 S D 0 ?4	9
7 14±S D 0 56	15 38±S D 1 07	2 2± S D 0 06	9
16 00±S D 1 50	32 34±S D 2 38	? 3±S D 0 15	8

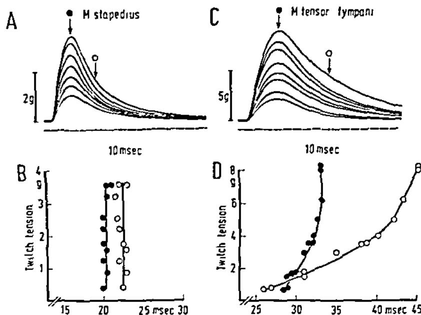


Fig 4 Isometric twitch contractions produced by stimulation of the appropriate motor nerve with increasing stimuli up to supramaximal strength of (A) the stapedius and (C) the tensor tympani of the cat In the diagrams in B (m stapedius) and D (m. tensor tympani) the contraction time (filled circles) and the half relaxation time (open circles) have been plotted against the twitch tension The plotted measurements are from individually recorded contractions not from the superimposed series in A and C

these small fibres have a higher threshold to electric stimulation (Eccles *et al* 1958), a gradual increase of the stimulus strength will be expected to excite first the fast motor units and later the slower ones Unfortunately a free-dissection of a sufficient length of the nerves to allow bipolar stimulation proved impossible, and a unipolar stimulation of the nerve *in situ* was employed In this case other factors such as the proximity of the different nerve fibres to the stimulating cathode also affected their excitability Nevertheless in the stapedius muscle such a gradual increase of the stimulus strength gave a gradual increase in the twitch tension, but did not affect the contraction and relaxation phase Fig 4 A

The plotting in Fig 4 B shows that the contraction time (filled circles) and the half relaxation time open circles re largely independent of the twitch tension as would be expected in a purely fast m scle

On the other hand a gradual increa e f th stimulus strength gave an increase in the tensor tympani twitch tension with a c rr nding increase of both the contraction time and the relaxation phase Fig 4 C D These findings were consistent with the idea that the tensor tympani contai a fast and a slow muscle component Pressure on the nerve trunk and cutting of bdivisions of the nerve to the tensor tympani were tried without success in an mpt to isolate one or the other of these assumed components

When recording the tetanic tension of the muscles the stimulus frequency was varied systematically in each muscle The tension at apparent fusion was taken as the maximal tetanic tension of the muscle In the cat apparent tetanic fusion was obtained at a stimulus rate of 75—100 cps in the tensor tympani and at a stimulus rate of 100—125 cps in the stapedius In the rabbit apparent fusion was obtained at a stimulus rate of 100—125 cps in both muscles

Discussion

Contraction force of the middle ear muscles

In the present study the maximal force of both the stapedius and the tensor tympani muscles was found to be considerably greater than observed in previous investigations In cats Wever *et al* (1955) found the maximal force of the tensor tympani to average 4 4 grams and of the stapedius to average 1 6 g In rabbits Wersall (1958) found the maximal force of the stapedius to average 8 62 g One probable reason for the low values found by Wever *et al* (1955) is their use of a stimulus frequency of 1000 cps which is unphysiologically high

The applied resting tension may also have been too low judging fom the data obtained during the present investigation The difference between the results ob tained by Wersall (1958) and the present results might also be due to a difference in the resting length and tension used Wersall (1958) determined the resting length of the muscle by estimating the natural position of the muscle tendons by the aid of the microscope However in view of the highly critical optimal value of the resting length of the stapedius (Fig 1 B) this method may not have a sufficient accuracy

The critical influence of the resting length of the stapedius muscle upon its twitch tension (Fig 2 B) probably reflects the highly isometric working conditions of this muscle With an intact ossicular chain the movements of the stapes during contrac tions of the stapedius muscles are maximally 50 μm (Philip 1932) Correspondingly deviation from the optimal length of little more than 50 μm gave large reductions of the tensions obtained (Fig 1 B)

Comparing the present data with the results of the tension studies of Wever and Bray (1937 1942) the tensor tympani of the cat when contracting maximally should be capable of reducing the amplitude of cochlear microphonic potentials (1000 Hz) from an initial value of 30 to 3 μV The sound intensity is thereby re duced by a factor of 20 dB ($20 \log \frac{30}{3}$ 20 dB)

Similar correlations of the data for the stapedius muscle of the cat revealed that this muscle should be capable of reducing the cochlear potential amplitude from 30 to 1 5 μV corresponding to a factor of 26 dB ($20 \log \frac{30}{1\ 5}$ 26 dB

It is well known that in the cat and in the rabbit both tympanic muscles contract in response to loud sounds Since there is no simple summation of the mechanical action of the two muscles it is not possible to calculate how much their combined

contraction will reduce the sound transmission. However, since each of them has been calculated capable of reducing the sound transmission by more than 20 dB, it is consequently not necessary to postulate any special structural arrangement of the muscle fibres to account for their action (Wever *et al.* 1955).

In rabbits, Møller (1965) found that single twitches of each middle ear muscle separately gave a reduction in the sound transmission of a low frequency tone (500—700 cps) of approximately 13 dB and that simultaneous contraction of both muscles gave a sound reduction of the same frequencies of 16—17 dB. Since the maximum force of these muscles under tetanic stimulation has been found to be 2.2 times stronger than the twitch, it seems safe to conclude that the middle ear muscles of the rabbit are capable of reducing the sound transmission to the same order of magnitude as that found in the cat.

Strong acoustic stimulation of the contralateral ear has previously also been used as stimulus when measuring the maximal force of the muscles. In decorticated rabbits and in cats under light pentobarbital anesthezia (Lorente de No 1935, Wersall 1958) the tension during maximal reflex contraction was up to 10 times weaker than the muscular tensions obtained in the present study upon supramaximal electrical tetanic stimulation. This difference can hardly be accounted for by the difference in recording methods alone. It is probable that the middle ear muscles on the ipsilateral side cannot be brought to maximal contraction by acoustic stimulation of the contralateral ear alone. This conclusion is supported by the findings of Møller (1962) who compared the impedance changes of the ear resulting from the contraction of the tympanic muscles upon acoustic stimulation of the ipsilateral and contralateral ear in cats and found the reflex threshold to be higher and the impedance changes to be smaller when stimulating the contralateral ear.

One important function of the middle ear muscles to which little attention has been paid is the problem of distortion during the transmission of sound vibration from one auditory ossicle to the next. If the contact pressure between two vibrating bodies is smaller than the accelerating force acting during the vibration, the two bodies will separate. In the ear the elastic ligaments are able to exert a sufficiently great pressure if the vibrations are small, but for stronger vibrations distortion is likely to take place unless the contact pressure between the vibrating ossicles is increased. Bekesy (1960) assumed that the maximum acceleration forces in the middle ear for which no distortion should occur are of the order of 20 g (19 600 dyn/cm²). From this he assumed that the tensor tympani muscle must be capable of producing a force of this magnitude. This prediction is supported by the present findings. The capacity of the middle ear muscle to exert strong muscular tensions is, consequently, also necessary to ensure a high fidelity transmission of loud sounds up to the pain threshold.

Contraction time of the middle ear muscles

The present investigation has shown that both the middle ear muscles in rabbit and cat are fast muscles. The data for the stapedius muscle are in accordance with the

findings of Wersall (1958) who also found a contraction time of the stapedius muscle in rabbit of about 20 ms

The longer contraction time of the tensor tympani found by Wersäll (1958) may have been caused by a reduced local temperature since in his experiments the middle ear was exposed to air at room temperature

If a muscle contains both a fast and a slow component, the relative strength of the two components will determine to which extent the contraction time and the half relaxation time will be prolonged due to the contribution of the slow component

In the caput mediale of the triceps brachii the slow component contributed so much to the total twitch that a gradual increase in the stimulus strength increased the contraction time from 30 5 to 59 ms and the half relaxation time from 85 to 120 ms (Eccles *et al* 1958) On the other hand the small component of slow muscle fibres in the intercostal muscles (Andersen and Sears 1964) and in the flexor carpi radialis (Gordon and Phillips 1953) did not affect the contraction and half relaxation time since these values are as would be expected in purely fast muscles

In the tensor tympani the slow component contributed much to the force of the last part of the twitch This slow component should therefore consist of ordinary twitch muscles and exist in addition to the more 'primitive slow muscle fibres with graded contraction such as Erulkar *et al* (1964) claim to have found in the tensor tympani, and which according to the anatomical investigations of Fernand and Hess (1969) should exist in both the tensor tympani and the stapedius muscles

I wish to thank dr Per Andersen for his encouragement and for his valuable help and criticism

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Tension and Contraction Time of Motor Units of the Middle Ear Muscles in the Cat

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Abstract

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The all or none contraction of 46 individual tensor tympani motor units and of 44 individual stapedius motor units in response to threshold stimulation of their motor neurones have been recorded. The tensor tympani was found to contain two types of twitch motor units, a fast type and a slow type. The fast type had a contraction time between 23 and 40 ms, a half relaxation [illegible] pe had a contra[illegible] ms and a tetani[illegible] twitch motor u[illegible] from 15 to 50 ms and a tetanic fusion rate between 75 and 100 Hz. The number of motor units was estimated as the ratio between the average twitch tension of the muscles to supramaximal stimulation and the average twitch tension of individual motor units. On the average the tensor tympani contained 92 fast and 40 slow twitch motor units and the stapedius 87 fast twitch motor units.

According to most theories on middle ear function the stapedius and the tensor tympani muscles, being attached to each end of the ossicular chain of the middle ear, are thought to have a synergistic action and an identical function (for reviews see Wever and Lawrence 1954, Simmons 1964). However during a recent investigation (Teig 1971) the tensor tympani was shown to consist of both a fast and a slow twitch muscle component, whereas the stapedius appeared to be a purely fast muscle. Since a difference in motor unit composition is thought to reflect a difference in function (Henneman and Olson 1965) this observation, if correct, would indicate a functional differentiation of the two middle ear muscles. The first purpose of the present investigation was to test this hypothesis. Contractions of a number of individual motor units in each muscle were recorded to see if the muscles were qualitatively different or not. The tensor tympani proved to consist of two types of twitch motor units as opposed to the stapedius which consisted of one type only. Further

more, by determining the tension which each motor unit can produce compared with that of the whole muscle a measure of the total number of motor units in these muscles was obtained. This figure is necessary to discuss the degree to which the tension of these muscles can be varied.

Methods

Operative procedure

Adult cats (2.2—4.5 kg) were anesthetized with sodium pentobarbital, tracheotomized and fixed in a head holder. The middle ear was widely exposed and the tensor tympani or the stapedius muscle was attached to an electro-mechanical transducer. This part of the procedure has been described in more detail previously (Teig 1971). The nerves to the muscles were left intact. The posterior part of the skull was then exposed and the posterior part of the cerebellum was removed by suction exposing the floor of the fourth ventricle. Using the obex as a reference (Berman 1968) stimulating electrodes held in a stereotactic manipulator could be placed in the motor nucleus of the trigeminal nerve (10 mm rostral 3.5—4.5 mm lateral to obex, at a depth of 0.5—2 mm from the floor of the ventricle) and in the facial nucleus (6 mm rostral, 3.5—4.5 mm lateral of obex, at a depth of 5—6 mm from the floor of the ventricle).

Stimulation. Thin insulated tungsten electrodes (tip diameter about 10 μm) were used as a cathode and inserted in the facial nucleus or in the motor nucleus of the trigeminal nerve depending on whether the stapedius or the tensor tympani muscle was tested. The anode was placed on the neck musculature which was exposed during the operation. When a preliminary localization of the appropriate nucleus had been obtained stereotactically, negative square pulses of 0.2 ms duration and an amplitude sufficient to elicite a twitch contraction of the corresponding middle ear muscle were applied. When the stimulation electrode was moved carefully and the size of the muscle twitch to a given stimulus intensity was observed simultaneously, the location of the motor nuclei of either the stapedius or the tensor tympani muscle could be determined accurately. With a proper stimulus strength an all-or-none contractile response of a single motor unit could be elicited. When the stimulating electrode was moved without changing the strength of the stimulus, the single motor unit response disappeared. Usually no response was seen until the electrode was moved 100 to 150 μm when a new all-or-nothing response could be elicited which, judging from its shape and size, was due to another motor unit.

Recording. Isometric contractions of the muscles were recorded by an electromechanical transducer (RCA 5734) in a special housing (Teig 1971). The output of the transducer was initially balanced out in a Wheatstone's bridge. A force applied to the tip of the transducer caused an imbalance of the bridge giving an output of 180 to 200 mV/g, varying from one transducer to the next. With the amplification necessary for studying contractions of single motor units of the middle ear muscles the background mechanical noise, mainly caused by the heart beat, distorted the signal. This was counteracted by averaging fifty to two hundred contractions. To ensure that the computed average represented the all-or-nothing response of a single motor unit a control of the input to the computer was required. The signal was digitalized by a voltage-to-frequency converter and fed into an electronic counter which was controlled by a gating pulse, thus allowing the tension signal to be integrated for 5 ms at the peak of the contraction. The integrated value was printed out and later plotted (see Fig. 1 C, F). Series in which more than one motor unit were activated, as indicated by more than one contraction level in the plot, were discarded.

Results

Since the tension and time relations of the muscle twitch are critically dependent upon the resting length of the middle ear muscles (Teig 1971) the optimal muscle length was first determined by applying a supramaximal stimulus to the appropriate nerve and measuring the resulting twitch at different muscle lengths. The contraction of the individual motor units were recorded at the optimal length for the contraction of the whole muscle.

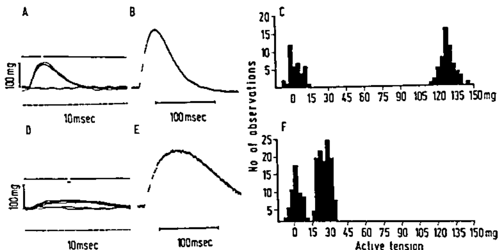


Fig 1 M tensor tympani A All-or none contractile response of a fast motor unit to threshold stimulation B Computed average of the contraction of the same motor unit in response to 96 consecutive stimulations C Histogram of the tension developed by the same motor unit to just threshold stimulation (57 successes and 39 failures) This unit had a twitch tension of 130 mg D All or none contractile response of a slow twitch motor unit to threshold stimulation E Computed average of the contraction of the same motor unit in response to 149 consecutive stimulations F Histogram of the tension developed by the same motor unit to just threshold stimulation (103 successes and 46 failures) This unit had a twitch tension of 27 mg

Contraction time

The all or none contraction of 46 individual tensor tympani motor units from 9 muscles and of 44 individual stapedius motor units from 6 muscles in response to threshold stimulation of their motoneurones have been recorded Fig 1 A shows the all or nothing response of a fast twitch tensor tympani motor unit The upper trace gives the gating pulse which was placed corresponding to the peak of the contraction This 5 ms pulse controlled the counter which gave the integrated value of the tension of a single contraction or failure over this period such as those plotted in C

Fig 1 B gives the computed average of 96 consecutive trials In Fig 1 C is plotted a histogram of the tension developed by the same motor unit to just threshold stimulation (57 successes and 39 failures) In this histogram the peak to the left represents the number of stimulations which did not elicit any contraction (failures) thus giving the baseline variation only (mostly due to the mechanical noise whereas the peak to the right represents the number of stimulations which resulted in the contraction of this particular motor unit (successes) The distance between the two peaks in the histogram consequently corresponds to the twitch contraction force of the motor unit which in this case was 130 mg Fig 1 D shows the all or nothing response of a slow twitch tensor tympani motor unit to just threshold stimulation of this motoneurone The corresponding computed average of 149 trials is given in

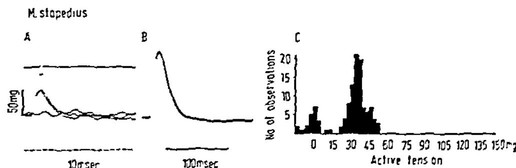


Fig 2 M stapedius A All-or-none contractile response of a stapedius motor unit B Computed average of the contraction of the same motor unit to 118 consecutive stimulations C Histogram of the tension developed by the same motor unit to just threshold stimulation (95 successes and 23 failures) The twitch tension of this unit was 36 mg

Fig 1 E, and a histogram of the corresponding tensions developed (103 successes and 46 failures in Fig 1 F This unit had a twitch tension of 27 mg

Using the same technique and way of display, Fig 2 A shows the response of a stapedius motor unit to just threshold stimulation of its motoneurone Fig 3 B gives the computed average of 118 trials These are also shown in the corresponding histogram (Fig 2 C) which represents 95 successes and 23 failures The twitch tension of this unit was 36 mg

In order to show how the different types of motor units were distributed in the tensor tympani the contraction time for all 46 motor units studied from this muscle were pooled in a histogram (Fig 3 A), and similarly for the 44 units of the stapedius muscle (Fig 3 B The hatched columns represent values obtained during a single experiment The distribution of the speed of contraction of the tensor tympani motor units was clearly bimodal with peaks around 28 and 75 ms Therefore, the motor

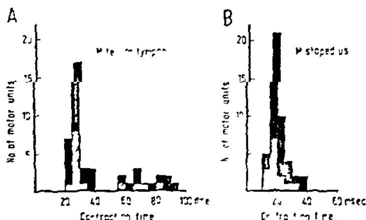


Fig 3 Histogram of the contraction time of 46 pooled tensor tympani motor units (A) and 44 pooled stapedius motor units B The hatched columns represent values obtained during a single experiment

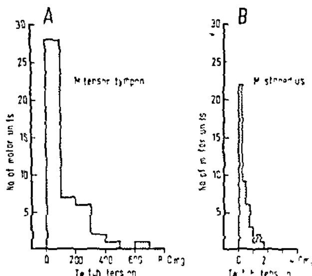


Fig 4 Histogram of the twitch tensions of fast and slow tensor tympani motor units taken together (A) and of the twitch tensions of the stapedius motor units (B)

units of this muscle can be divided into a fast twitch and a slow twitch group based upon their contraction times One group, represented by the motor unit in Fig 1 A, B, C, was composed of units having twitch contraction times ranging from 23 to 40 ms with half relaxation times from 17 to 46 ms The other group represented by the motor unit in Fig 1 D, E, F, comprised units with contraction times between 58 and 92 ms with half relaxation times ranging from 52 to 100 ms

In contrast, the distribution of the contraction time of the stapedius motor units (Fig 3 B) was unimodal, but skewed to the left with a peak around 30 ms Based on the temporal characteristics the motor units of this muscle thus represent one group only The contraction time ranged from 14 to 39 ms and the half relaxation time from 15 to 50 ms

Twitch tension

In the tensor tympani the twitch tension of the fast motor units ranged between 28 and 640 mg and the slow twitch motor units between 16 and 34 mg Fig 4 A is a histogram of the twitch tensions of fast and slow tensor tympani units taken together The twitch tension of the stapedius motor units ranged between 11 and 168 mg The histogram in Fig 4 B shows the distribution of the twitch tensions of the motor unit sample from this muscle The distribution in both Fig 4 A and 4 B is skewed to the left indicating that there was in both muscles a predominance of units which developed small tensions with a progressive decrease in number of stronger units In order to see whether there was any correlation between the speed and size of contractions of single motor units, the contraction time (Fig 5 abscissa) has been plotted against the twitch tension (ordinate) of individual motor units in the tensor tympani (A) and the stapedius muscles (B) The twitch tensions of the slow tensor tympani

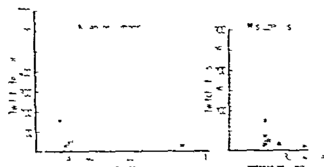


Fig. 5. Contraction time (abscissa) plotted against the twitch tensions of individual motor units in the tensor tympani (A) and in the stapedius muscles (B).

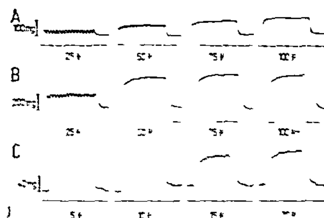


Fig. 6. Response to repetitive stimulation at various stimulus frequencies of (A) a single stapedius motor unit (same unit as in Fig. 3 A, B), (B) of a single fast tensor tympani motor unit (same unit as in 2 A, B) and (C) of a slow twitch tensor tympani motor unit. Time base 100 ms.

mo[illegible] were all small, considerably smaller than the mean tension of the fast motor units but the two groups cannot be separated from each other by virtue of the difference in twitch tension alone. Both in the tensor tympani and the stapedius muscle the [illegible] motor unit had a faster contraction time than the average of the group. Also in the stapedius the slowest motor units were all weak.

Tetanus and te[illegible]

Fig. 6 shows the responses to repetitive stimulation at 25, 50, 75, and 100 Hz of a single stapedius motor unit (A) (same unit as in Fig. 2 A, B) and of a single fast tensor tympani mo[illegible] (same as in Fig. 2 A, B). In all such experiments the fusion rate of the [illegible] unit was between 100 and 150 Hz and of the fast tensor tympani m[illegible] between 75 and 100 Hz. Fig. 6 C shows the response of a slow twitch tenso[illegible] unit to repetitive stimulation at 5, 10, 15 and 20 Hz. At the sens[illegible] required the background mechanical noise interfered with the tension curve. H[illegible] appeared to occur at a stimulus rate of 15–20 Hz. To obtain maxim[illegible] tension it was necessary with a longer stimulation period than that used in t[illegible] hence the difference in sweep length).

With the present experimental arrangement and the stimulation employed tetanic stimulation at a strength intended to excite one motor unit only, often recruited another unit making a pure contraction curve for a single motor unit difficult to obtain For this reason, few data on the tetanus/twitch ratio were obtained When judging from the record only one motor unit contributed to the tetanus in 4 recordings from the stapedius motor units and in 3 recordings from the fast tensor tympani motor units In these motor units the tetanus/twitch ratios varied between 2 8 and 4 2 In the slow twitch tensor tympani motor unit group only one such value was obtained with a ratio of 3 0

Discussion

Comment on the sample of motor units

The 46 tensor tympani and the 44 stapedius motor units were stimulated after random penetration of the respective nuclei with the stimulating electrode The cells giving origin to the nerve to the tensor tympani are located in the posteroventral part of the motor nucleus of the trigeminal nerve (Szentagothai 1949) Cells in this region are medium sized and more densely packed than the larger cells of the dorsal part of the nucleus (Berman 1968) The cells giving origin to the stapedius nerve are of small size and are located in the dorsomedial group of the facial nucleus (Vraa Jensen 1942) Since each middle ear muscle thus apparently are innervated by motoneurones of relatively even size and distribution random penetration by the stimulation electrode should not lead to a selection of any special motoneurone type However the method of threshold stimulation might favour the excitation of fast motor units since slow motor units because of their smaller size have a higher threshold to electric stimulation than fast ones (Eccles Eccles and Lundberg 195[illegible]) This relation also holds for small specialized muscles (Appelberg and Emonet-Denand 1967) For this reason the percentage of fast motor units in the sample from the tensor tympani might be too high and the possibility that the stapedius muscle might contain a few slow twitch motor units which failed to be elicited because of adjacent fast motoneurones with lower electric thresholds cannot be excluded

Types of twitch motor units in the middle ear muscles

The present investigation has shown that the tensor tympani contains two types of twitch motor units a slow type and a fast type In contrast with the previously mentioned in the first paragraph of the Discussion the stapedius [illegible] contains only one type which is fast

This myographic demonstration of two types of twitch motor [illegible] in the tensor tympani corresponds to the electromyographical findings of [illegible] Sato [illegible] Kirikae (1954) who described two types of firing patterns of motor units [illegible] tensor tympani in the cat One type discharged with long [illegible] charge interval of 60—80 ms and showed no adaptation [illegible] stimulation of the contralateral ear The other type discharged with [illegible]

minimal interval of 40—50 ms with a high voltage and showed marked adaptation to continuous acoustic stimulation. A discharge interval of 60—80 ms, which corresponds to a firing frequency of 15—16.7 Hz, would be sufficient to give complete fusion of the slow twitch motor units (Fig. 6 C). The observed minimal discharge interval of 40—50 ms of the fast twitch units, corresponding to a firing frequency of 20—25 Hz, however, would hardly give any fusion of these units. From the data on tetanic fusion (Fig. 6 B) one would expect a maximal firing frequency of more than 50 Hz. To the knowledge of the author there are no reports on the minimal discharge interval in individual stapedius motor units. From the data on tetanic fusion (Fig. 6 A) one would expect a maximal firing frequency of more than 75 Hz.

Force produced by individual motor units

The distribution of the tension of the motor units in the tensor tympani and the stapedius muscles clearly showed a predominance of units which developed small tensions. This skewed distribution is similar to that found in the gastrocnemius, in the soleus and in the flexor digitorum longus of the cat (Wuerker, McPhedran and Henneman 1965, McPhedran, Wuerker and Henneman 1965, Olson and Swett 1966). The functional significance of this distribution pattern was discussed by Henneman and Olson (1965) who showed that the tetanic tensions or sizes of motor units were inversely related to the excitability of their motoneurones. They claimed that this inverse relationship between the size of a motor unit and the excitability of its motoneurone allows small tensions to be produced and precisely controlled by selective mobilization of a varying number of small motor units, of which many are available. Applied to the middle ear muscles this principle could explain the smooth increase in tension observed by Wersall (1958) in both the tensor tympani and the stapedius muscle in the rabbit during weak acoustic reflex stimulation of the contralateral ear. The longer contraction time of the smallest motor units in both muscles (Fig. 5) contributes to the smoothness of the contraction curve during weak reflex stimulation.

Number of motor units in each muscle

Unfortunately the number of motor units in the middle ear muscles has not been estimated with any certainty since the number of sensory fibres in their motor nerves is not known. The 44 motor units recorded from the stapedius muscles had an average twitch tension of 42.6 mg. The average twitch tension to supramaximal stimulation of these muscles was 3.7 g (Teig 1971). This gives an average of 87 twitch units in the stapedius muscle. In the sample from the tensor tympani muscles the 32 fast motor units had an average twitch tension of 112.0 mg and the 14 slow motor units an average twitch tension of 26.4 mg. This should be related to the average twitch tension of 13.6 g in these muscles (Teig 1971).

The average contraction time of the whole muscle was 29 ms while the slow units had an average contraction time of 72 ms. Since the slow twitch motor units will

have reached only about half their maximal tension 29 ms from the onset of contraction, the slow twitch motor units in the sample contribute with half their peak force to the measured total twitch tension Making allowance for this, the tensor tympani muscle should consist of 132 twitch motor units, 92 of these being of the fast and 40 of the slow variety

Blevins (1964) found the stapedius nerve of the cat to consist of an average of 715 nerve fibres In the tensor tympani he found an average of 1123 nerve fibres (Blevins 1963) The present data indicate that only about 12 per cent of the nerve fibres to the middle ear muscles supply twitch motor units Since in most species few muscle spindles have been found in these muscles (Steinitz 1907, Winckler 1959, Malmfors and Wersäll 1960 a, 1960 b) and since they even are claimed to be non-existent in the cat (Blevins 1963, 1964), the remaining nearly 90 per cent of the nerve fibres would be sensory This percentage of afferent fibres may sound high, but should be related to the results of Boyd and Davey (1966) who found the proportion of afferent fibres in the nerves to various hindlimb muscles to vary as much as from 34 to 95 per cent

However, there is electrophysiological evidence for a tonic extrafusal fibre system in the tensor tympani of the cat (Erulkar *et al* 1964) Fernand and Hess (1969) have described muscle fibres in both middle ear muscles with multiple nerve terminals and other morphological characteristics typical of slow muscle fibres upon stimulation do not exhibit propagated action potentials and do not twitch Some of the fibres to be accounted for may be nerve fibres supplying non twitch musculature However, the support for the theory that the middle ear musles contain non twitch fibres is circumstantial and needs verification with more direct methods

Although there is little support for the existence of polyneuronal innervation of individual muscle fibres in regular body musculature (Brown and Matthews 1960), this possibility cannot be ruled out in the case of the middle ear muscles As pointed out by Cooper (1965), cranial muscles may be completely different from regular body muscles Therefore, a comparison between the two groups may be misleading Such a polyneuronal innervation would give a too small estimated number of motor units in the middle ear muscles

Function and electrophysiological properties

The two middle ear muscles are often considered together when reference is made to their function However, the difference between their twitch motor unit composition indicates that the action of the two muscles is complementary rather than identical A similar difference has been found between two other synergistic muscles of the body The gastrocnemius muscles has been found to contain both fast and slow motor units (Wuerker *et al* 1965, Burke 1967) whereas the soleus muscle contains only slow motor units (McPhedran *et al* 1965) In their discussion on the significance of this difference between the two muscles, Henneman and Olson (1965) conclude that the segregation of different properties in the gastrocnemius and in the soleus gives them qualities which could not be met by a single muscle

The length tension diagram showed that the stapedius was capable of exerting a high tension only within a very narrow working range (Teig 1971) The present finding, that the stapedius contains fast motor units only, further indicates that this muscle is highly specialized, able to produce strong isometric tension quickly These qualities might relate to its function as the principal phase shifter of the middle ear, since, according to Simmons (1964), a swift and continual shift of the resonant peaks of the middle ear is necessary to avoid resonances and at the same time retain the sensitivity of the ear

Spontaneous rhythmic activity in the tensor tympani with relative little activity in the stapedius has been recorded in the cat during slow circling movements and also without any associated body or head movements or any apparent eliciting stimulus (Carmel and Starr 1963) Such longlasting activity, which might relate to the maintenance of auditory attention (Simmons 1964) offers one explanation for the existence of both fast and slow twitch motor units in the tensor tympani, since slow twitch motor units are konwn to be more resistant to fatigue than fast ones However, the exact significance of the slow twitch units in relation to the function of the tensor tympani is at present not clear

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Estimation of Alveolar P_{O_2}

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Abstract

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dependent regional differences in alveolar P_{O_2} From simultaneous measurements of arterial P_{CO_2} it is inferred that the end-expired P_{O_2} approximates the time and volume weighted mean of alveolar P_{O_2} only at high rates of exercise breathing air

Alveolar P_{O_2} values are conventionally given as either the calculated mean alveolar (effective) P_{O_2} (Riley *et al* 1946 Bjurstedt *et al* 1962 Jones *et al* 1966 Rosenhamer 1967 Whipp and Wasserman 1969), or the measured end expired (end tidal) P_{O_2} (Severinghaus Stupfel and Bradley 1957 Bartels *et al* 1955 Lahiri *et al* 1971, Laszlo *et al* 1971) Theoretically however several factors affect these two alveolar P_{O_2} values differently, e g body position regional distribution of ventilation in the lungs, and expiratory flow rate In spite of the fact that rapidly responding oxygen analyzers using mass spectrometry or fuel cells have been available for some years there seems to be no systematic study of the relationship between the end expired and mean alveolar P_{O_2} values on the one hand and the arterial P_{O_2} on the other Such measurements were undertaken in the present study by using a recently developed, simple method for measuring P_{O_2} with a rapidly responding O_2 electrode sampling respired air (Friesen and McIlroy 1970 Healthy subjects were studied at rest in the lying and sitting positions breathing either air or low oxygen mixtures Comparisons were also made at two different work loads in the sitting position

TABLE I Dimensional and functional data

Subject	Age (yrs)	Sex	Height (cm)	Weight (kg)	$\dot{V}_{O_2max}$* (l/min)	Heart rate exercise breathing air (beats/min)	
						Work load I	Work load II
1	29	M	170	66	3 6	96	138
2	23	M	178	70	2 1	108	180
3	19	F	170	57	1 8	124	190
4	30	M	177	66	2 8	96	156
5	27	F	168	59	1 9	120	192
6	32	M	183	68	3 1	90	148
7	26	F	168	79	2 4	114	156
8	25	M	174	71	2 2	114	174
9	25	F	160	53	1 8	120	180
10	24	M	179	73	2 4	102	168
11	40	M	173	88	2 4	114	168
12	33	M	180	77	3 6	78	138

* From nomogram of Åstrand (1960)

Methods

F_{CO_2} with an infrared analyzer The P_{O_2} and P_{CO_2} record... cent deflec...

...cuits according to Nunn *et al* (1965) Mean alveolar P_{O_2} was calculated using the formula (Riley *et al* 1946)

$$P_{A_{O_2}} = P_{I_{O_2}} - P_{a_{CO_2}} \left[F_{I_{O_2}} + \frac{1 - F_{I_{O_2}}}{R_E} \right]$$

Procedure

Each subject was first studied resting in the supine position Measurements were made during air breathing and then after the subject had breathed 12 % O in nitrogen for 15 min Expired gas was collected for 3 min and 1 min, respectively Arterial samples were taken in duplicate during the periods of gas collection The subject next sat up and measurements were repeated at rest and during exerc...

as before

The meas...

...all after 15 min of hypoxia

TABLE II Average values breathing air (n = 12)

	Arterial P_{O_2} mm Hg	Arterial P_{CO_2} mm Hg	Mean alveolar P_O mm Hg	End expired P_{O_2} mm Hg	End-expired P_{CO_2} mm Hg	Mean alveolar—arterial P_{O_2} mm Hg	End expired—arterial P_{O_2} mm Hg	End expired — mean alveolar P_{O_2} mm Hg	Arterial — end expired P_{CO_2} mm Hg
Resting supine	95 3 ±6 8	40 2 ±0 7	100 2 ±4 9	103 2 ±5 7	38 5 ±0 8	4 8** ±4 7	7 8*** ±5 7	3 0** ±3 1	1 7** ±2 0
Resting sitting	94 0 ±4 0	37 3 ±0 9	99 1 ±5 4	106 0 ±6 5	33 2 ±1 1	5 1*** ±3 5	12 0*** ±4 2	6 9*** ±4 2	4 1*** ±1 5
Work load I sitting	95 0 ±4 4	39 7 ±0 4	101 1 ±3 1	103 1 ±3 3	38 1 ±0 5	6 1*** ±3 8	8 1*** ±4 1	2 0 ±3 7	1 6*** ±1 0
Work load II sitting	95 9 ±7 8	35 2 ±0 9	110 9 ±3 8	110 8 ±4 0	36 3 ±1 1	15 0*** ±5 2	14 9*** ±6 3	0 1 ±2 3	−1 1* ±1 4

*** P< 0 001
** P < 0 01
* P < 0 05

Results

The means and standard deviations of the values obtained during air breathing are [illegible] in Table II, together with the average individual differences and their standard deviations Statistically significant differences with P values < 0 05 are *indicated in the table In all circumstances the differences between both end expired* and arterial, and mean alveolar and arterial P_{O_2}'s were significant Both these differences were smallest when resting supine and largest at the highest rate of work At rest the end expired P_{O_2} values were greater than the mean alveolar values (P < 0 01), and more so sitting than supine The differences between end expired to mean alveolar P_{O_2} values during exercise were not statistically significant (P > 0 05) Both values increased with exercise at the highest load, the change in arterial P_{O_2} was small Pa_{CO_2} showed a tendency to decrease with the highest load, suggesting the occurrence of metabolic acidosis Resting sitting the arterial P_{CO_2} value was 4 mm higher than the end expired value (P < 0 001) Statistically significant arterial to end-expired P_{CO_2} differences though small, were also present when resting supine, and at the lower but not at the higher work rate

The values while breathing 12 % O_2 are shown in Table III 4 subjects felt faint at rest in the sitting position, and no values were obtained in this situation End expired to mean alveolar P_{O_2} differences were not significant at rest, either sitting or supine During exercise the end expired P_{O_2} was significantly smaller (4 mm Hg P < 0 005) than the mean alveolar value The differences at rest between end expired and arterial P_{O_2} with 12 % O_2 were smaller than those during air breathing, but the mean alveolar to arterial differences were about the same The largest end expired

TABLE III Average values breathing 12 % O_2

	Arterial P_{O_2} mm Hg	Arterial P_{CO_2} mm Hg	Mean alveolar P_{O_2} mm Hg	End-expired P_{O_2} mm Hg	End-expired P_{CO_2} mm Hg	Mean alveolar—arterial P_{O_2} mm Hg	End-expired—arterial P_{O_2} mm Hg	Endexpired—mean alveolar P_{O_2} mm Hg	Arterial—end expired P_{CO_2} mm Hg
Resting supine n = 12	43.7 ±3.6	36.5 ±0.7	50.3 ±4.6	48.5 ±4.9	34.6 ±0.8	6.6*** ±2.9	4.9* ±4.3	−1.8 ±3.8	1.9*** ±1.0
Resting sitting n = 7	46.6 ±8.9	34.2 ±1.4	49.5 ±6.7	50.5 ±6.8	32.2 ±1.5	4.9* ±4.4	5.9* ±3.1	1.0 ±1.9	2.0* ±1.2
Work load I sitting n = 11	41.9 ±3.1	34.1 ±0.7	54.7 ±3.3	50.8 ±3.7	34.0 ±0.9	12.8*** ±2.9	9.0*** ±4.8	−3.9* ±3.1	0.1 ±1.0

*** $P < 0.001$
* $P < 0.05$

to arterial and mean alveolar to arterial P_{O_2} differences were present during exercise The differences between arterial and end-expired P_{CO_2} averaged 2 mm Hg at rest, both sitting and supine No significant P_{CO_2} difference was present during exercise

Discussion

At rest during air breathing, the end-expired P_{O_2} was higher than the calculated mean alveolar value, implying that the P_{O_2} at the end of expiration was higher than the time weighted mean of the alveolar P_{O_2} during the respiratory cycle This finding appears at first sight unexpected in view of the continuous alveolar gas exchange, which causes the P_{O_2} in each alveolus to fall steadily during expiration If all parts of the lungs empty simultaneously as suggested by Sikand, Cerretelli and Farhi (1966), then the end-expired P_{O_2} would conceivably be lower than the mean alveolar P_{O_2} A possible explanation for the present finding is that uneven distribution of alveolar ventilation (for review, see Permutt 1966) might result in an early emptying during expiration of alveoli in which P_{O_2} is low due to uneven distribution of V/Q ratios and that end-expired gas therefore, comes preferentially from alveoli with high P_{O_2} values The observations of Dollfuss, Milic Emili and Bates 1967) and of Millette *et al* (1969) that during expiration in the erect posture the upper lung regions, where the P_{O_2} is likely to be higher than in the lower regions Fowler and Reed 1961, West 1962) empty most rapidly towards the end of expiration tallies with this hypothesis Analogously, the finding that the average difference between end expired and mean alveolar P_{O_2} was higher in the sitting than in the supine position (Table II) would be compatible with the demonstration by Ball *et al* (1962) and Bryan *et al* (1964) that V/Q distribution from apex to base is most non uniform in the upright posture

As a corollary to the above reasoning, the observation of a reduction with exercise of the end-expired to mean alveolar P_{O_2} difference, both breathing air (Table II) and 12 % O_2 (Table III) is compatible with findings by Bashoff, Ingram and Schilder (1967) and Millette *et al* (1969) which indicate that upper lung regions empty earlier during expiration with increased expiratory flow rates. Furthermore, regional alveolar ventilation should be more evenly distributed during exercise (Bryan *et al* 1964), and $\dot{V}/Q$ inequalities be reduced (*cf* West 1962, Rosenhamer 1967), which should diminish any effects of sequential alveolar emptying. The more complete washout of the kinetic dead space during exercise (Lilly 1946) should likewise tend to lower the end-expired relative to the mean alveolar P_{O_2}, thus reducing the difference present at rest between these two values.

Exercise presumably exaggerates the cyclic changes in alveolar gas composition with each breath, because the rate of oxygen uptake and carbon dioxide production increases (Riley 1946, Yamamoto 1960). This factor has been used to explain the well established fact that during exercise, contrary to rest, the end-expired P_{CO_2} may be higher than the mean alveolar or arterial P_{CO_2} (Matell 1963, Rahn and Farhi 1964, Table II). In the present exercise experiments a corresponding "reversed" difference for P_{O_2}, i. e. a lower end-expired than mean alveolar value, was demonstrated during low oxygen breathing, but not during air breathing (Table II and III, exercise values). This discrepancy between the air and low oxygen experiments may be explained as due to various effects of hypoxia, such as more even distribution of ventilation (Arborelius and Lilja 1969), reduction of perfusion inequalities throughout the lungs (Dugard and Naimark 1967), reduction of end-capillary to arterial P_{O_2} differences due to perfusion inequality (Perkins, Adams and Flores 1956) and larger tidal volumes with higher expiratory flow rates.

The observation of an increased arterial to end-expired P_{CO_2} difference following the change from the supine to the upright body position at rest during air breathing (Table II) agrees with earlier findings by Bjurstedt *et al* (1962). The increase can be attributed to the effect of gravity which results in a reduced perfusion of the upper lung regions (West and Dollery 1960, Bryan *et al* 1964), so that part of the ventilation is wasted (*cf* Rahn and Farhi 1964). Wasted ventilation leads *per se* to an increased arterial to end-expired P_{CO_2} difference (Severinghaus and Stupfel 1957), and this effect would be exaggerated by late emptying of upper lung regions with low P_{CO_2} (Read 1966). It is obvious that in the upright sitting position at rest with an arterial to end-expired P_{CO_2} difference of 4 mm Hg (Table II) the calculated "mean alveolar P_{O_2}", which is derived from arterial P_{CO_2}, can not represent the composition of an "ideal" alveolar sample, i.e. equal the time and volume weighted mean of the alveolar P_{O_2} (alveolar P_{O_2} integrated over the respiratory cycle and the different lung regions). The use, in this situation, of the deviation of the "mean alveolar" P_{O_2} from arterial P_{O_2} as a measure of the combined effects of venoarterial shunt and "$\dot{V}/Q$ inequalities" (*cf* Mellemgaard 1966) would accordingly be misleading since wasted alveolar ventilation would not significantly affect this difference.

At the highest rate of work, breathing air, it was found not only that the average

end-expired to "mean alveolar" P_{O_2} difference was insignificant, but also that the average P_{CO_2} difference between the end-expired gas and arterial blood was negligible. Although the relative work load (in per cent of $\dot{V}_{O_2max}$, see Table I) varied considerably between individuals, this observation seems to indicate that at high levels of exercise the end-expired O_2 and CO_2 pressures approximate those of an "ideal" alveolar gas sample (*cf.* p. 26).

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Acta physiol. scand. 1972. 84 29—33

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Correlation between Hyperinsulinemia and Hyperphagia in Rats with Ventromedial Hypothalamic Lesions

By

B E. HUSTVEDT and A LØVØ

Received 16 June 1971

Abstract

HUSTVEDT, B E and A LØVØ *Correlation between hyperinsulinemia and hyperphagia in rats with ventromedial hypothalamic lesions* Acta physiol scand 1972 84 29—33

Plasma levels of immunoreactive insulin (IRI) and glucose were determined in unrestrained rats equipped with chronically implanted venous catheters Electrolytic lesions were then placed bilaterally in the ventromedial hypothalamic area Food intake was restricted in order to prevent hyperphagia A 60—280 % increase in the IRI level with a concomitant small increase in blood glucose was demonstrated on the second day post-operatively The animals were then given free access to food The gain in body weight during the following 3 days of *ad lib* feeding and the post-operative increase in the IRI level of each animal was positively correlated

Bilateral destruction of the ventromedial hypothalamic area (VMH of rats produces hyperphagia and extreme obesity (Hetherington and Ranson 1939) Elevated plasma levels of immmunoreactive insulin (IRI) have been reported in animals with such lesions (Hales and Kennedy 1964 Frohman and Bernadis 1968) The etiology of this hyperinsulinemia has not yet been established

Firm knowledge of the plasma IRI and blood glucose changes during the first post operative days is essential for a fruitful investigation of this problem Moreover it is important that the nutritional state of lesioned animals remains virtually unchanged during the observation period as both over and underfeeding will influence plasma IRI and blood glucose levels In order to obtain blood samples under comparable and possibly basal conditions before and after the lesions were made it would seem advantageous to implant venous catheters permanently into a suitable vessel Such an arrangement would be of particular value during the post operative period since lesioned animals are aggressive and more difficult to handle than normal rats

The investigation reported here was undertaken in order to study the early effects of VMH lesions on fasting plasma levels of insulin and glucose from unrestrained

animals under conditions during which hyperphagia was prevented. Furthermore, we have attempted to establish whether changes in these parameters might in some way be correlated to the body weight gain of the individual animals on a subsequent *ad lib* feeding schedule.

Material and Methods

Male Wistar rats (AF/Han/Mo/Han) weighing 240—280 g were used. They were provided with a chronically u ... 1969). The catheter (Silastic medical gr ... ed into the external jugular vein under ... rization the animals were kept in separa ... from the catheteri zation. A modest loss of body weight took place during the first two days post operatively. Then the food intake was normalized and the body weight increased again.

Stereotaxically guided lesions (David Kopf instruments Model 900) were produced by electrolysis 7 days after catheterization. An anodal d.c. current (1.5 mA, 15 s) was passed through steel electrodes of even thickness (0.4 mm), insulated by varnish except for 0.5 mm at the tip. The stereotaxic coordinates used for destroying the ventromedial area of hypothalamus were 6.0 mm anterior to the inter aural line, 0.6 mm lateral to the midline and 1.0 mm above the ventral floor of the skull.

The animals were offered 15 g of food daily for the first 5 days post operatively which equals the average food intake of normal rats of the same size. After this initial period they were fed *ad l b* as before. The food intake was recorded daily and the animals were weighed every 3 days. Only the body weight was recorded during the *ad lib* feeding period of the lesioned animals because it proved difficult to obtain reliable measurements of the food intake.

Blood samples were obtained 5 days and 2 days before the VMH destruction and again 2 days after the operation. The sampling was performed on unrestrained animals 8 h after withdrawal of food. No handling of the animals took place during the last 2 h prior to sampling and Heparin was not infused. Blood samples (2 ml) were drawn into heparinized syringes and immediately placed in ice water. Duplicate aliquots of 25 μl each were secured for the glucose alysis. The rest of the blood was centrifuged and plasma separated from the cells. The sma was frozen and kept at −25° C for later analysis. Immediately after sampling the rats ceived transfusions of heparinized fresh donor blood.

Analytical procedures

The blood glucose concentration was determined in 25 μl of whole blood. A commercial glucose oxidase reagent set was used (GLOX, Kabi). Plasma IRI was assayed in samples of 50 μl in quadriplicate according to Ørskov (1967). A proinsulin free preparation of crystal line rat insulin (Batch R 169, Novo) was used as standard. Plasma IRI concentrations were calculated by means of the computer program developed by Volund *et al.* (1970) adapted for use on the CDC 3300 computer of the University of Oslo. The computer output consists of a table of estimates with statistical confidence limits for each unknown, the variation between replicates of the unknowns and standards being taken into account.

Results

The plasma IRI concentrations were increased considerably 2 days after VMH destruction compared to the level before the operation in all animals tested. This is demonstrated in Fig. 1. There was, however, a greater variation in IRI values after lesion than before, 38—107 μU/ml 2 days after lesion as compared to 18—37 μU/ml 2 days before lesion.

The IRI levels before VMH destruction also increased somewhat from the first to the second measurement. We believe that the concomitant improvement in the nutritional state of the animals as they regained their appetite after catheterization is the most likely explanation for this observation. The average food intake on the

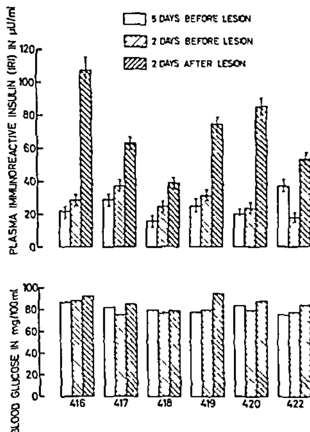


Fig 1 Blood glucose and plasma immunoreactive insulin (IRI) levels in rats before and after the placement of ventromedial hypothalamic lesions The vertical bars in the IRI diagram represent the 95 % confidence interval

day of the first blood sampling was 8—10 g, whereas it had risen to 13—15 g 3 days later, when the second blood sample was taken This corresponds closely to the average food intake of normal rats of this size Thus the IRI level measured 2 days before VMH destruction is probably representative for normal rats of this age after 8 h of fasting

The blood glucose level 2 days before the lesions were produced was in the range of 75—80 mg/100 ml for all except one of the animals As the concentration of blood glucose is markedly influenced by stress, the uniform level that was observed seems to confirm our impression that the blood sampling procedure was performed under comparable conditions in the different animals All animals had a slightly increased blood glucose level 2 days post-operatively compared to the values measured 2 days before the operation

In Fig 2 the individual increases in the fasting IRI level post-operatively is plotted against the subsequent gain in body weight of each animal during 3 days of *ad lib* feeding The first day with free access to food is not included in this period in order to avoid the initial body weight increase due to the rapid filling of the digestive tract

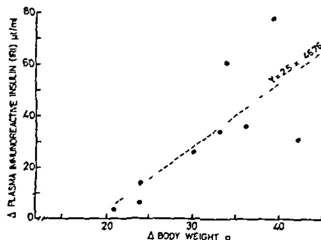


Fig 2 The relation between the increase in plasma immunoreactive insulin (IRI) from 2 days before to 2 days after lesion, and the increase in body weight following 3 days feeding *ad lib* Each filled circle represents values from one animal The linear regression line is supplied

which takes place when hyperphagic animals are fed *ad lib* There is a reasonably good positive correlation between the 2 sets of values The correlation coefficient equals 0 74 with a 95 % confidence interval of 0 23—0 93 The correlation is significant, $p < 0.02$ All animals used in Fig 2 exhibited varying degrees of hyperphagia as their body weights increase considerably more than 1—2 g/day which is the average weight gain for normal rats of this age

Discussion

The present work shows that the plasma IRI level is considerably elevated already 2 days after VMH destruction in adult rats even when hyperphagia is prevented Furthermore this IRI elevation is found to be positively correlated to the subsequent gain in body weight of each animal after 3 days of *ad lib* feeding

Hales and Kennedy (1964) observed increased fasting IRI concentrations in lesioned animals after 7 days of *ad lib* feeding but not as early as on the second postoperative day They therefore attributed the hyperinsulinemia to the hyperphagia and obesity that accompanies the VMH lesions in adult rats It is well documented indeed that obesity leads to adaptive metabolic changes including an elevated IRI level (Karam Grodsky and Forsham 1963) Hyperinsulinemia has also been reported in voluntarily hyperphagic humans Sims *et al* 1968 However Frohman *et al* (1969) found increased IRI levels on the 4th post operative day in lesioned weanling rats which they claim develop obesity without hyperphagia Recently Han and Frohman (1970) reported hyperinsulinemia in hypophysectomized rats 5 days after VMH destruction as compared to hypophysectomized control animals Both groups were tubefed equal amunots of food They concluded therefore that hyperinsulinemia may develop after VMH lesions are produced in the absence of both hyperphagia and pituitary hormones

Our results extend the previous evidence for an early development of hyperinsulinemia in lesioned rats Probably the elevated IRI level is established even

before the second post-operative day. In one single animal we have sampled blood 24 h post-operatively and found increased IRI concentration. Furthermore we have confirmed that the hyperinsulinemia is not a result of adaptive metabolic changes following hyperphagia. The early onset of hyperinsulinemia after VMH destruction may indicate a direct nervous or humoral action on the endocrine pancreas causing increased insulin secretion. Alternatively, the operation may lead to other nervous or endocrine disturbances that result in insulin resistance of some tissue(s). In that case the elevated IRI level would be a secondary effect of such disturbances.

Normal rats given regular injections of longacting insulin exhibit increased food intake and accumulation of body fat (MacKay, Callaway and Barnes 1940). It seems reasonable to expect that endogenous hyperinsulinemia will have the same effects. The first hypothesis is therefore an attractive one because it provides a simple explanation for the hyperphagia and obesity of lesioned animals. The correlation reported in this paper appears to support this hypothesis although it does not prove any causal relationship between the hyperinsulinemia and the subsequent hyperphagia.

If the primary effect of VMH lesions is increased insulin secretion, hypoglycemia in the fasting state post-operatively would be expected. This is not the case, however. The blood glucose is in fact slightly elevated compared to the level 2 days before the operation. In our opinion this finding supports the alternative hypothesis of insulin resistance caused by VMH destruction. However, a firm conclusion as to the primary effect of such lesions cannot be drawn on basis of the presented results alone.

We thank Dr. Henrik Ege, Novo Industri A/S, for a gift of crystalline rat insulin and Miss Åse Turter, the Hormone Laboratory, Aker Hospital, Oslo, for valuable assistance with the insulin radioimmunoassay.

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Acta physiol. scand. 1972 84 34—39

From the Department of Pharmacology, Karolinska Institutet, Stockholm Sweden

Effects of Malate and Oxaloacetate in Canine Subcutaneous Adipose Tissue

By

BERTIL B FREDHOLM

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Abstract

FREDHOLM, B B *Effects of malate and oxaloacetate in canine subcutaneous adipose tissue* Acta physiol scand 1972 84 34—39

The effect of malate and oxaloacetate infusions was studied in the isolated canine subcutaneous adipose tissue It was found that oxaloacetate (4—7 mM) reduced the venous lactate/pyruvate ratio whereas malate (6—12 mM) increased it During and following sympathetic nerve stimulation approximately equimolar amounts of glycerol and free fatty acids (FFA) were released When oxaloacetate was infused the amount of FFA released increased whereas the glycerol release was essentially unchanged Malate on the other hand reduced the FFA release in spite of an essentially unchanged glycerol release Both anions caused an increased uptake of glucose The changes in re esterification induced were similar to that produced by the lactate/pyruvate couple The results are taken as circumstantial support for the concept that the degree of re esterification can be altered with the intracellular red ox state

The release of free fatty acids (FFA) from isolated canine subcutaneous tissue perfused with blood *in situ* is increased by electrical stimulation of the nerve supply (Rosell 1966 Fredholm 1970) This effect was antagonized by lactate in concentrations above 7 mM in the arterial blood (Fredholm 1971) Since the concomitant release of glycerol was not affected by lactate the effect was considered due to an increased re esterification This mechanism of action of lactate was also suggested by Miller *et al* (1964 Dihydroxyacetonephosphate and pyruvate compete for the reducing equivalents in NADH in skeletal muscle (Peterson *et al* 1961) which suggests that lactate might cause increased re esterification by producing more *a* glycerolphosphate secondary to an increased cytoplasmatic NADH/NAD ratio (Miller *et al* 1964) The finding that pyruvate apparently decreased re esterification (Fredholm 1971) is in accordance with this scheme as is the finding that lactate production and re-esterification increased in parallel during prolonged nerve stimulation (Fredholm and Karlsson 1970)

Not only are the lactate/pyruvate and *a* glycerolphosphate/dihydroxyacetone phosphate equilibria dependent upon the cytoplasmatic NADH/NAD ratio, but this

appears to hold true also for the malate/oxaloacetate equilibrium in the cytoplasm (Bucher and Klingenberg 1958) It was therefore considered to be of interest to study the effects of infused malate and oxaloacetate on the release of FFA and glycerol, as well as the ratio of lactate and pyruvate in venous blood in the isolated canine subcutaneous adipose tissue

Methods and Materials

The experiments were conducted on 14 fed female mongrel dogs weighing between 8 and 18 kg (mean 12 kg) Anesthesia was induced with sodium pentobarbital (25 mg/kg i v) and was supplemented with additional doses of 25—50 mg i v whenever necessary during the course of the experiment

Abdominal subcutaneous adipose tissue was isolated from all surrounding tissues including skin as described in detail by Rosell (1966) The tissue thus prepared weighed between 16 and 55 g (mean 35 g) After cannulating the artery to the tissue with a plastic tube free flow perfusion was started blood was led via a drop-counter (Lindgren 1958) from the femoral artery The venous outflow from the adipose tissue was returned to the animal via the femoral vein Coagulation was prevented by heparin (Vitrum) 2500 I U /kg BW i v given 1—2 h before the start of the experiment Systemic arterial blood pressure was measured in the femoral artery and recorded together with the blood flow on a Grass polygraph

The mixed nerve to the adipose tissue was cut between ligatures and placed on a bipolar silver electrode Approximately rectangular pulses of supramaximal intensity (12 V) and duration (2 msec) *were delivered at the rate of 4 cps from a Grass stimulator for a period of* 10 min

Venous samples were taken at 15 and 5 min before nerve stimulation, during and 5, 15 and 25 min after stimulation The venous samples drawn were of 6—8 ml blood The time ... mpling and ... there was a ... min before, ... one block ... mM) and oxaloacetate (4—7 mM) infusion It has been shown earlier that two consecutive control blocks are for all practical purposes identical (Fredholm 1971)

Oxaloacetic acid (Boehringer, Mannheim) and l malic acid (Calbiochem Luzern) were neutralized to pH 7 4 by 1 M NaOH and dissolved in saline of such concentration to assure approximately isoosmolarity of the infusion solution Infusions were given via a side branch in the arterial drop-counter by means of a motor driven syringe that delivered the infuson solution at a *rate of 0 05—0 09* ml/min

... ere determined in blood using commercial reagents ... ples ... rell ... [ct) ... be ... sue ... was found by subtracting ... after stimulation by the

Results

The composition of the arterial blood is given in Table I The values agree well with those reported earlier for heparinized blood (Fredholm and Karlsson 1970) There were no significant differences in the arterial blood composition during infusion of saline, malate or oxaloacetate

The uptake of glucose, lactate and pyruvate in canine subcutaneous adipose tissue has been found to be dependent upon the arterial concentration (Fredholm 1970,

TABLE I Composition of arterial blood in 14 dogs (mean ± S E , mM)

Glucose	5 4 ±0 90
FFA	0 56±0 05
Glycerol	0 13±0 02
Lactate	1 37±0 23
Pyruvate	0 06±0 01

TABLE II The net release of FFA and glycerol during infusion of oxalocetate (4—7mM) and malate (6—12 mM) compared to control in the same dogs (mean ± S E , μmol min/100 g adipose tissue) Figures within parenthesis denote number of experiments The p value is based on a t test for paired variates

	FFA	Glycerol
Control	0 12±0 06	0 17±0 04
Oxaloacetate (7)	0 11±0 05	0 19±0 04
Control	0 03±0 14	0 33±0 10
Malate (8)	0 11±0 14	0 17±0 7 $p < 0.05$

1971, Fredholm and Rosell 1970) Such a relationship was not found for FFA and glycerol, however The present findings agree with those earlier reported

The net release of FFA in unstimulated adipose tissue was not changed by oxaloacetate or malate administration, as seen from Table II Similarly, the net release of glycerol under these conditions were unaffected by oxaloacetate On the other hand the rate of glycerol release during malate infusion was lower than during the control period in the same dogs (Table II) There is no ready explanation for this, but it can be noted that the rate of glycerol release in this control group was higher than that in the oxaloacetate controls

The FFA mobilization upon nerve stimulation was elevated during oxaloacetate administration and diminished during infusion of malate The effects of oxaloacetate are shown in Fig 1, which illustrates a typical experiment During nerve stimulation the blood flow decreased afterwards there was a marked poststimulatory hyperemia During this hyperemic period there was a clearcut increase in the rate of glucose uptake This pattern of response was only seen in 4 out of 13 dogs, however General ly nerve stimulation does not alter the rate of glucose uptake (Fredholm 1970, Fred holm and Karlsson 1970 Fredholm and Rosell 1970) During and following a control nerve stimulation (saline infusion) approximately 28 μmol/100 g glycerol and 17 μmol/100 g FFA was released During oxaloacetate the glycerol release amounted to 25 μmol/100 g and the FFA release to 48 μmol/100 g After oxaloacetate the values were 19 and 10 μmol/100 g, respectively Thus nerve stimulation during saline infusion induced a larger release of glycerol than of FFA, whereas the con verse was true for nerve stimulation during oxaloacetate infusion Assuming that glycerol is not utilized by canine subcutaneous adipose tissue to any appreciable ex-

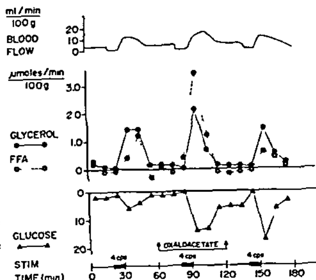


Fig 1 The effect of infusing 8 µmol/min oxaloacetate on blood flow FFA and glycerol release and on glucose uptake The nerve was stimulated with 4 cps 12 V 2 msec for 10 min Adipose tissue (25 g) isolated from a 10 kg dog anesthetized with 25 mg/kg Nembutal Hct 42 per cent

tent this means that the esterification of fatty acids is decreased during oxaloacetate infusion

The effects of malate or oxaloacetate on glycerol and FFA release following sympathetic nerve stimulation as well as on glucose uptake and venous lactate/pyruvate ratios are summarized in Fig 2 The standard errors in the figure refers mainly to differences between individuals which were large in confirmation of earlier results (Fredholm 1970) For this reason the hypothesis tested by Student s t test was that the uptake or release of a metabolite during oxaloacetate or malate infusion was identical to 100 per cent of the same parameter during saline infusion

Both malate and oxaloacetate increased the glucose uptake significantly, or by 76 ± 22 and 151 ± 47 per cent respectively There was also a tendency towards an increased mobilization of glycerol upon nerve stimulation which was insignificant, however The FFA release following nerve stimulation increased by 132 ± 45 per cent during oxaloacetate infusion and decreased by $39 + 13$ per cent during malate infusion The ratio between venous lactate and pyruvate decreased during the infusions of oxaloacetate and during malate it decreased

Adipose tissue blood flow was $5\,9 \pm 1\,4$ ml/min/100 g which is within the normal range reported earlier (Fredholm 1970 Ngai Rosell and Wallenberg 1966) It increased following both oxaloacetate (24 ± 7 per cent) and malate ($?5 \pm 9$ per cent) There was no indication that the increase in vascular resistance induced by nerve stimulation was altered by either of the anions

Discussion

The amount of FFA released following nerve stimulation was lower during malate infusion than during the corresponding control The glycerol release was somewhat

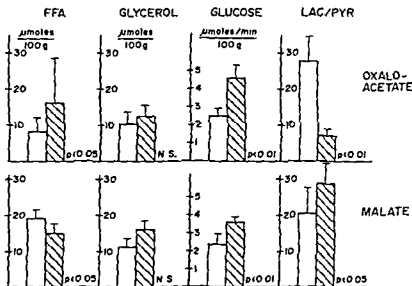


Fig 2 The net increase in glycerol and FFA release caused by nerve stimulation calculated as described in Methods as well as the rate of glucose uptake and the venous molar lactate/pyruvate ratio during infusion of saline (unfilled bars) oxaloacetate (n = 6) and malate (n = 7) (Hatched bars) Means ± S E For further details see text

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although not significantly increased This can be taken as evidence of increased re esterification of fatty acids during malate infusion Similarly the evidence indicates decreased re esterification of fatty acids during oxaloacetate infusion Thus the oxidized and reduced members of a cytoplasmatic red ox couple had opposite actions *on the re esterification This finding conforms with the hypothesis* discussed above that re esterification might be dependent upon the cytoplasmatic NADH/NAD ratio

The uptake and utilization of malate and oxaloacetate was not examined in this study and the effects of these anions on the adipocyte intermediary metabolism could not be determined In particular there is no direct evidence that the cytoplasmatic NADH/NAD ratio was altered

It was found however that malate increased the ratio of lactate/pyruvate in the venous blood from adipose tissue whereas oxaloacetate had the opposite effect The plasma and cytoplasmatic lactate/pyruvate ratios are generally assumed to be coupled (Alpert 1965) The present finding is thus in line with the idea that cytoplasmatic lactate/pyruvate and malate/oxaloacetate equilibria are coupled via the NADH/NAD system (Boxer and Devlin 1961 Bucher and Klingenberg 1958) It seems possible, therefore that malate increased the cytoplasmatic NADH/NAD ratio and oxaloacetate *reduced it*

Obviously the administration of these extremely high concentrations of Krebs cycle intermediaries will affect the metabolism of the tissue in many ways The increase in glucose uptake is one example of this It is therefore necessary to interpret the

present findings with caution Yet it is safe to conclude that the findings do not contradict the hypothesis formulated earlier (Fredholm 1970, 1971) that changes in re esterification can be brought about by alterations in the cytoplasmatic red-ox state and that this might be a way of rapidly modifying the mobilization of lipids from adipose tissue

The present investigations was supported by Grants from the Swedish Medical Research Council (B72 40X 2553 04) and Karolinska Institutet
The skilful technical assistance of Miss Gunilla Wikberg is gratefully acknowledged

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Acta physiol. scand 1972. 84. 40—47

From the Toxicology Laboratories, AB Astra Sodertalje, Sweden

The Tissue Distribution of ^{3}H-Terbutaline (Bricanyl®) in Mice

By

N O Bodin, E Hansson, C H Ramsay and Å Ryrfeldt

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Abstract

Bodin, N O E Hansson, C H Ramsay and Å Ryrfeldt *The tissue distribution of ^{3}H terbutaline (Bricanyl®) in mice* Acta physiol scand 1972 *84* 40—47

The tissue distribution of ^{3}H terbutaline a new β stimulaing tagent was investigated in mice by whole body autoradiography as well as by determinations of unchanged and biotransformed terbutaline in serum lung heart and liver

The radioactivity was localized mainly to the extracellular space and the levels in most tissues were of the same magnitude as that in blood no uptake and storage of the drug was observed Neither terbutaline nor its metabolites penetrated the blood brain barrier or the placenta barrier High relative concentrations of unchanged drug were found in the tissues studied especially in the lung A low rate of biotransformation is considered to be the cause for this

Terbutaline is a new β receptor stimulating agent with action predominantly to tracheal and bronchial muscle The drug is clinically active after oral application and has a long duration (Persson and Olsson 1970)

While adrenaline and noradrenaline have been the subject of many studies concerning the uptake and storage in tissues relatively few studies of synthetic β receptor stimulating agents have been performed Hertting (1964) studied the fate of ^{3}H isoprenaline in the rat and Butler Moser and Kot (1969) determined its distribution volume in the isolated lung of the dog Dengler (1966) reported some data on the distribution of ^{3}H orciprenaline

The aim of the present study was to investigate the tissue distribution of ^{3}H terbutaline in mice by whole body autoradiography as well as by determinations of unchanged and biotransformed terbutaline is some tissues

Materials and Methods

Compound

Tritiated dl terbutaline hydrobromide [1 (3 5-dihydroxyphenyl 2 t butylamino 1 ^{3}H-ethanol hydrobromide] with a specific activity of 1 0—1 5 mCi/mg was obtained from the Research Laboratories Astra Lakemedel AB Sweden The radiochemical purity was tested by TLC on

silica gel plates (D C Fertigplatten Kieselgel E Merck AG) and the plates were developed with cyclohexane — isopropanol — conc ammonia (5 13 2) The radioactivity on the chromatograms was detected with the aid of a radiochromatogram scanner (Packard Model 7200) Only one radioactive peak could be detected and this peak had the same Rf value (0 27) as reference terbutaline in this system

Autoradiography

Eight male mice and 3 pregnant mice of the NMRI strain were injected into a tail vein (25 mg/kg and 10 m[illegible] mg/kg) ^{3}H terb[illegible] 0 01 ml/g b w [illegible] tively in the 3 g[illegible]

in 1 % aqueous solution of carboxymethylcellulose and deepfrozen for 10 min at −70° C. Sagittal sections (30 μm) at different levels of the whole body were cut at −10° C and each section was attached to tape (No 810, Minnesota Mining and Manufacturing Co) and dried at the same temperature for 2—3 days The tape mounted sections were pressed against Ilford G-5 nuclear emulsion plates and stored at −20° C for exposure during 6—8 weeks The plates were developed in Ilford ID 19 for 5 min at 22° C and fixed in Kodak F5

Tissue levels of terbutaline

Animal experiments

Male mice of the NMRI strain weighing 20—25 g received 1 mg/kg of terbutaline hydrobrom[illegible]

with an amount of water equal to twice the weights of the tissue sample These homogenates were used for assay of total radioactivity and of radioactivity attributable to unchanged terbutaline

Analytical methods

The total radioactivity of the tissues was obtained by dissolving 100 mg of serum or tissue [illegible] n peroxide The samples [illegible] ntillation solution (7 0 g [illegible] was added The samples [illegible] ter Model 3320 The counting efficiency was estimated by the external standard channels ratio procedure and the counting was performed in a way to avoid an error larger than ±3 % (95 % confidence limit) in the total count of each sample

Unchanged terbutaline was extracted by ion pair extraction An aliquot of serum or tissue homogenate (200 mg) was diluted with 1 00 ml distilled water followed by 0 20 ml perchloric acid (10 %) After centrifugation 1 00 ml of the supernatant was removed by aspiration and transferred to a glass-stoppered test tube containing 1 00 ml 1 mol l Na phosphate buffer solution pH 7 0 equilibrated with 1 % w/v DEHPA [di ethylhexyl phosphoric acid] in methylene chloride and 0 050 ml 3 mol l NaOH This water phase was extracted by gentle shaking with 2 00 ml methylene chloride containing 0 5 % w/v DEHPA The phases were separated b[illegible] by [illegible]

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samples it was further concluded that 0 07 of the biotransformed terbutaline was coextracted into the organic phase The values for tissue concentrations obtained were corrected accordingly

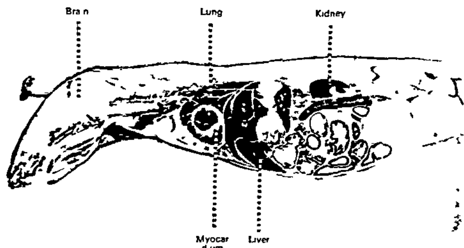


Fig 1 Autoradiogram showing the distribution of radioactivity (light areas) in a mouse 5 min after i.v. injection of ^{3}H terbutaline hydrobromide

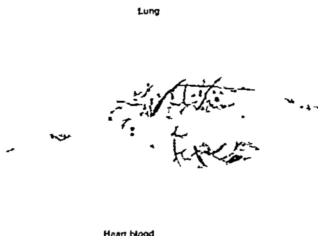


Fig 2 Autoradiogram showing the distribution of radioactivity (light areas) in a mouse 15 min after i.v. injection of ^{3}H terbutaline hydrobromide

Results

Autoradiographic studies

The distribution pattern of radioactivity at various times after administration as revealed by autoradiography is shown in Fig 1—4

Intravenous administration

5 min after injection (Fig 1 high concentration of radioactivity was seen in the blood The concentration in the liver gall bladder and kidneys exceeded the blood level The concentration in the lung subcutaneous connective tissues and salivary gland was approximately the same as in blood Moderate amounts of radioactivity

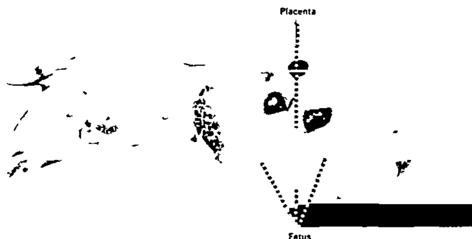


Fig 3 Autoradiogram showing the distribution of radioactivity (light areas) in a pregnant mouse 1 h after i v injection of ^{3}H terbutaline hydrobromide

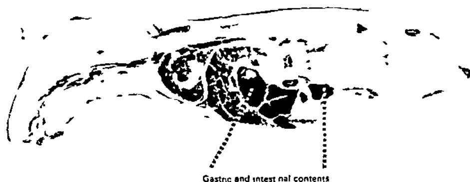


Fig 4 Autoradiogram showing the distribution of radioactivity (light areas) in a mouse 30 min after oral administration of ^{3}H terbutaline hydrobromide

were seen in *e g* thymus, brown fat myocardium spleen and adrenals No activity was seen either in the brain or in the spinal cord

15 min after administration (Fig 2) the distribution pattern was mainly the same as after 5 min However very large amounts of radioactivity could be seen in parts of the intestinal contents and the urinary bladder

30 min after injection the distribution pattern was similar to that found in the 5 and 15 min experiments Very high concentration of activity could be seen in the kidneys urinary bladder, gall bladder and parts of the intestinal contents The pregnant mouse showed high concentration of activity in the liver and placenta No activity could be seen in the fetus

1 h after injection a decrease of activity could be seen in most tissues compared with the activity seen at the shorter survival times, but still very high concentration could be seen in the gall bladder, intestinal contents and urinary bladder. Moderate amount of activity was seen in the heart blood. Moderate amounts were also seen in the lung, connective tissue, skin, and salivary gland. The pregnant mouse showed moderate levels of activity in the liver and placenta. No activity was seen in the fetus (Fig. 3).

4 h after administration very high concentration of activity was only seen in the intestinal contents and urinary bladder. Only a low amount of activity was seen in the liver of the pregnant mouse. No radioactivity could be seen in the placenta and fetus.

8 h after injection only traces of activity could be seen in the intestinal contents and urinary bladder. After 24 h all activity had left the animal.

Oral administration

30 min after administration (Fig. 4) high level of radioactivity was seen in the blood. The concentration in the urinary bladder, stomach contents, gall bladder, kidneys, and intestinal contents was higher than in the blood. The lung, liver, and subcutaneous connective tissue showed approximately the same activity as the blood. Only small amounts of activity could be seen in e.g. thymus, brown fat, myocardium, spleen and adrenals.

1 h after administration the distribution picture was quite similar to that found in the 30 min experiment. The heart blood still showed a moderate amount of activity and the activity in the other tissues seemed to be at the same level as at the shorter survival time.

4 h after administration very large amounts of activity were seen in the gall bladder, urinary bladder and intestinal contents. Radioactivity was also seen in the stomach contents. No radioactivity could be seen in the body tissues.

8 h after administration very large amounts of activity were seen in the urinary bladder, stomach contents and intestinal contents.

24 h after administration activity could only be seen in the intestinal contents and after 48 h no activity could be seen in the animal.

Tissue levels of drug after [illegible] administration

Tissue levels of total radioactivity [illegible]d as unchanged drug) and radioactivity attributable to unchanged terbutaline [illegible] administration are given in Table I. The time course of the concentration of unchanged drug in the tissues studied is shown in Fig. 5.

The serum levels of total radioactivity were about the same at 5 and 15 min after injection but the 1 h value was about 1/7 of the preceding values. The amount of unchanged drug was gradually diminishing. At 5 min after injection about 1/4 of the total activity was attributable to unchanged drug and 1 h after administration

[illegible]

Tissue	Concentration (µg/g wet weight)					
	5 min		15 min		60 min	
	total	unchanged	total	unchanged	total	unchanged
serum	1 23±0 14	0 29±0 03	1 21±0 09	0 12±0 01	0 19±0 11	0 02±0 01
liver	4 06±0 25	0 53±0 05	1 72±0 21	0 21±0 04	0 31±0 04	0 06±0 01
lung	0 67±0 05	0 33±0 03	0 68±0 03	0 23±0 03	0 17±0 03	0 08±0 01
heart	0 38±0 03	0 16±0 02	0 46±0 08	0 16±0 05	0 11±0 02	0 05±0 01

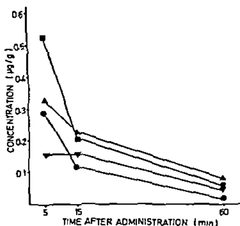


Fig 5 The time course of the concentration of unchanged drug after i v administration of ^{3}H terbutaline hydrobromide, 1 mg/kg b w, to male mice Each point represents the mean value of 5 pools, each including samples from 5 animals
● serum, ■ liver, ▲ lung and ▼ heart

about 1/10 of the serum activity was attributable to terbutaline

Of the tissues investigated the highest concentration at 5 min after injection was found in the liver where the total concentration of activity was about 4 times the serum concentration However, only about 1/8 of the liver activity was attributable to terbutaline Total radioactivity as well as radioactivity attributable to unchanged drug were gradually diminishing and the 1 h values were about 1/10 of the 5 min values

The concentration of total radioactivity in the lung was about half the serum radioactivity at 5 and 15 min after injection but the 1 h values for serum and lung tissue were nearly the same The amount of unchanged drug in the lung was consistently higher than the serum levels and remained high during the whole experiment

Although the time course of heart concentrations was comparable with that of lung, the heart concentrations of total radioactivity and activity attributable to unchanged drug were lower than lung concentrations

Discussion

The tissue distribution is of considerable interest in connection with studies of β adrenergic stimulating agents, since many studies (e g Axelrod Weil Malherbe and Tomchick 1959, Gryglewski and Vane 1970, Iversen and Whitby 1962, Whitby Hertting and Axelrod 1960) have shown that uptake and storage of catecholamines by various tissues may represent an important mechanism in the physiological disposition of these compounds

Hertting (1964) found that isoprenaline, like noradrenaline, disappeared rapidly from the vascular compartment However, only negligable quantities were found bound in the tissues Most of the activity present in the tissues 10 min after administration was in the form of the O methylated metabolite Thus, uptake and binding of unchanged isoprenaline do not play any important role in the fate of this substance

The results of the present study indicate that this statement applies also to terbutaline The radioactivity was localized mainly to the extracellular space both after oral and intravenous administration The levels in most tissues were of about the same magnitude as that of blood, with some exceptions Higher concentrations were found in the excretory organs These results seem to be in agreement with those reported by Dengler (1966) for orciprenaline a drug structurally similar to terbutaline This should indicate that these drugs and their metabolites have no tendency to be taken up by tissues other than liver and kidney

The fact that radioactivity was virtually absent in the central nervous system and in the fetuses indicates that neither terbutaline nor its metabolite(s) are able to penetrate the blood brain barrier or the placenta barrier to any appreciable extent

In contrast to the case with isoprenaline high relative concentrations of unchanged terbutaline were found in the tissues studied In view of the long duration of the bronchospasmolytic effect (Persson and Johnsson 1970) it is interesting to note that the concentration of unchanged terbutaline remained comparatively high in lung tissue during the whole experiment

The relatively high concentration of unchanged terbutaline in e g lung tissue is considered to be a consequence of a low rate of biotransformation Studies *in vitro* have shown that terbutaline being a dihydric phenol of the resorcinol type and possessing a tertiary butyl group at the nitrogen is not attacked by catechol O methyl transferase (COMT or by monoamine oxidase (MAO) (Persson and Persson, personal communication) The biotransformation observed is presumed to be a conjugation reaction with glucuronic acid or sulfuric acid

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Acta physiol. scand 1972. 84 48—53

From the Institute of Physiology, University of Oslo, Norway

The Volume Responses of Various Vascular Beds to a General Blood Loss. A Study Carried out in Rats Exposed to Two Different Procedures of Anesthesia

By

P Aarseth and H Piene

Received 22 June 1971

Abstract

Aarseth P and H Piene *The volume responses of various vascular beds to a general blood loss A study carried out in rats exposed to two different procedures of anesthesia* Acta physiol scand 1972 *84* 48—53

Several vascular compartments have been claimed to have marked blood depot functions. In the present experiments an attempt has been made to compare different vascular beds of the rat in this respect by evaluating the tissue blood content in bled and non bled animals. Erythrocytes and plasma albumin were labelled by radioisotopes and the whole rat was then immersed in liquid nitrogen The lungs the heart with the thoracic vessels the splanchnic organs and samples from skeletal muscles were then removed and their content of radioisotopes estimated In animals anesthetized intraperitoneally with pentobarbitone the pulmonary blood volume and the skeletal muscle blood content were markedly reduced after a blood loss of 12 % When however the animals were anesthetized by ether inhalation and by a subsequent small dose of pentobarbitone i v then a similar bleeding caused a marked reduction in the splanchnic blood volume but almost no reduction in the pulmonary blood volume or the skeletal muscle blood content

It has been demonstrated that the pulmonary blood volume in the rat is markedly reduced subsequent to a moderate blood loss (Aarseth 1970) The aim of the present investigation has been to compare the blood mobilizing role of the pulmonary vascular bed with that of some other vascular areas which usually are considered to have important blood depot functions The vascular beds in the splanchnic area and in skeletal muscle and the heart with the large intrathoracic vessels were included in the investigation Rats were given radioisotopically labelled erythrocytes and albumin i v , whereafter a moderate bleeding was performed The circulatory situation was then arrested by rapid freezing of the animals in liquid nitrogen (Everett Simmons and Lasher 1956) When groups of bled and non bled animals were compared, it was found that the prebleeding blood content as well as the depot function of the various vascular beds were markedly influenced by the procedure of anesthesia employed

TABLE I The effect of a standardized blood loss on the blood volume of various vascular beds in two groups of differently anesthetized animals Mean values ± S D p calculated according to Wilcoxon's two-sample test

Organ	Group I Anesthesia Pentobarbitone i p Free access to food beforehand			Group II Ether inhalation + subsequent small i v injection of pentobarbitone Food deprivation for 24 h before bleeding		
	Non bled animals n = 10	Bled animals (Mean blood loss 12 6 %) n = 10	Reduction in blood volume	Non bled animals n = 14	Bled animals (Mean blood loss 12 4 %) n = 14	Reduction in blood volume
Pulmonary blood volume (ml)	2 00 ± 0 42	1 69 ± 0 23	0 31 p = 6 %	1 59 ± 0 23	1 50 ± 0 17	0 09 p = 13 %
Pulmonary hematocrit	26 0 % ± 4 1	31 5 % ± 3 9		39 2 % ± 2 7	39 5 % ± 3 2	
Heart and large thoracic vessels blood volume (ml)	1 52 ± 0 26	1 45 ± 0 17	0 07	1 70 ± 0 18	1 69 ± 0 18	0 01
Splanchnic blood volume (ml)	2 92 ± 0 60	3 07 ± 0 68	—0 15	3 00 ± 0 73	2 39 ± 0 45	0 61 p = 1 1 %
Skeletal muscle blood content (ml/g)	0 021 ± 0 005	0 018 ± 0 005	0 003* p = 4 9 %	0 023 ± 0 003	0 022 ± 0 004	0 001

* From Aarseth 1970

Methods and results

Male Wistar albino rats were used The animals were rather homogenous as regards b w (210 ± 17 g), total blood volume (13 ± 1 5 ml) and large vessel hematocrit (47 % ± 1 5)

Total blood volume, tissue blood content and *tissue hematocrit* were evaluated on the basis of injection of radioactively labelled erythrocytes and albumin and subsequent analysis of radioactivity in blood and tissue samples The tissue samples were taken after rapid freezing of the animals in liquid nitrogen The methods used have previously been described in detail (Aarseth 1970)

The animals in one group (group I, 20 rats) were anesthetized with 30 mg/kg of pentobarbitone (Nembutal®, diluted 1 4 in saline) intraperitoneally This group had free access to food and water until they were used in acute experiments Each individual animal was immersed in liquid nitrogen 50 min after introduction of anesthesia and 10 min after injection of isotopes One half of the animals in this group were bled (mean blood loss 12 6 %) through a venous catheter during the last 5 min before freezing, the other half served as non bled controls The lungs, the heart with the intrathoracic vessels and the splanchnic organs (i e liver, stomach, intestine and mesentery, spleen not included) were then separately excised from the frozen animals, and the blood content of the three compartments calculated from their radioactivity

The results are shown in the left part of Table I The bleeding induced a marked reduction in pulmonary blood volume, whereas it resulted in small changes only in the blood content within the heart and the mediastinal vessels and in the splanchnic organs The pulmonary hematocrit increased after the bleeding

The animals in a second group (group II 28 rats) were deprived of food (but not of water) for the last 24 h before experimentation They were then kept anesthetized by ether inhalation during the introduction of an i v catheter, a procedure which lasted for 15—30 min When the femoral vein had been cannulated, ether anesthesia was withdrawn and just

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The Volume Responses of Various Vascular Beds to a General Blood Loss. A Study Carried out in Rats Exposed to Two Different Procedures of Anesthesia

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Several vascular compartments have been claimed to have marked blood depot functions. In the present experiments an attempt has been made to compare different vascular beds of the rat in this respect by evaluating the tissue blood content in bled and non-bled animals. Erythrocytes and plasma albumin were labelled by radioisotopes and the whole rat was then immersed in liquid nitrogen. The lungs, the heart with the thoracic vessels, the splanchnic organs and samples from skeletal muscles were then removed and their content of radioisotopes estimated. In animals anesthetized intraperitoneally with pentobarbitone the pulmonary blood volume and the skeletal muscle blood content were markedly reduced after a blood loss of 12 %. When, however, the animals were anesthetized by ether inhalation, and by a subsequent small dose of pentobarbitone i.v., then a similar bleeding caused a marked reduction in the splanchnic blood volume, but almost no reduction in the pulmonary blood volume or the skeletal muscle blood content.

It has been demonstrated that the pulmonary blood volume in the rat is markedly reduced subsequent to a moderate blood loss (Aarseth 1970). The aim of the present investigation has been to compare the blood mobilizing role of the pulmonary vascular bed with that of some other vascular areas which usually are considered to have important blood depot functions. The vascular beds in the splanchnic area and in skeletal muscle and the heart with the large intrathoracic vessels were included in the investigation. Rats were given radioisotopically labelled erythrocytes and albumin i.v., whereafter a moderate bleeding was performed. The circulatory situation was then arrested by rapid freezing of the animals in liquid nitrogen (Everett, Simmons and Lasher 1956). When groups of bled and non-bled animals were compared, it was found that the prebleeding blood content as well as the depot function of the various vascular beds were markedly influenced by the procedure of anesthesia employed.

nary blood volume was found to be just the same (2.0 ml) as that found in the pentobarbitone anesthetized rats which had had free access to food. Thus food deprivation did apparently not affect pulmonary blood volume.

An attempt was then made to evaluate the pulmonary blood content in non-bled unanesthetized animals. Injections of isotopes for blood volume measurements could not be carried out without anaesthesia. However, a very close correlation (r=0.94 for group I) appeared to exist between pulmonary blood volume and total lung weight. This made it possible to make a good estimate of the pulmonary blood volume in the unanesthetized, quickly frozen animals simply by weighing their excised frozen lungs. A series of 7 animals thus treated showed a lung weight of 2.32 g. In the animals of groups I and II the mean lung weights had been 3.1 and 2.4 g, respectively. Thus the pulmonary blood volume in unanesthetized animals seems to be close to that of animals treated with light ether anesthesia and subsequent i.v. injections of a small dose of pentobarbitone.

Discussion

Chien and Usami (1969) found a small reduction of central blood volume (*i.e.* the blood content between the right atrium and the abdominal aorta), but a more marked reduction in the splanchnic blood volume in moderately bled dogs (15 % blood loss). Dellenback and Muelheims (1960) found that during exsanguination of rats anesthetized with pentobarbitone (50 mg/kg i.p.) the largest contribution to the shed blood came from the liver (40 %), the heart and the lungs (20 %) and the skeletal muscles (17 %). Reynell *et al.* (1955) found a marked volume reduction of the splanchnic vasculature in dogs after a blood loss of 30 %. The splanchnic blood volume was reduced relatively more than that of the pulmonary vascular bed.

The present series of experiments have shown that the degree of post hemorrhagic depot function of various vascular beds in the rat depends on the procedure of anesthesia employed. In rats anesthetized with i.p. injected pentobarbitone, the vascular beds of the lungs and skeletal muscle appear to be the most important blood depots on a moderate blood loss. When the i.p. route of administration was avoided by using introductive ether inhalation and subsequent i.v. injection of a small dose of pentobarbitone, then the splanchnic vascular bed was much more important as a blood depot.

It is a generally accepted view (Price 1960) that ether inhalation cause marked activity in the sympathetic nervous system in most species. Barbiturates are said not to have such an effect. Aarseth (1971a) has shown that the marked reduction in pulmonary blood volume seen during hemorrhage in pentobarbitone anesthetized rats depends on intact sympathetic nerve fibres to the lung. In the ether anesthetized unbled animals of the present series of experiments increased discharge in sympathetic nerve fibres could have markedly reduced the pulmonary blood volume before any bleeding took place. In that case the pulmonary

tance vessels would have less additional blood to expel subsequent to a blood loss. However, lung weight and thereby probably also the pulmonary blood volume was equally low in unbled unanesthetized animals as in the unbled ether anesthetized rats Unanesthetized animals do not necessarily have a "normal" pulmonary blood volume which one could use as a reference value, though It is conceivable that the handling of these rats prior to freezing might have excited them and thereby resulted in increased discharge rate in sympathetic nerve fibres and reductions in pulmonary blood volume

Another explanation to the difference between the pentobarbitone- and the ether anesthetized nonbled rats is that the pulmonary blood volume of the former may be increased during the experiments The pentobarbitone anesthetized non bled rats did not only show a relatively large, but also a variable pulmonary blood volume This is reflected in the much larger standard deviation for pulmonary blood volume in this group than in the other group of animals (Table I) Observations by Aarseth (1971b) in pentobarbitone anesthetized rats made hypervolemic by 1 5 ml transfusions may be relevant to this variability He found a markedly increased lung blood volume and a reduced pulmonary hematocrit in these hypervolemic animals The anesthetized rats in the present series of experiments also received infusions (of heparin and isotopes) the volumes of which were not negligible (0 3—0 4 ml) These infusions may have caused similar changes of pulmonary blood volume and pulmonary hematocrit as in Aarseth s previous series The large variability of pulmonary blood volume in the present animals with i p pentobarbitone anesthesia might indicate that their lung vasculature has been in a labile state where the vascular volume would easily change in either direction If so this could again explain why during bleeding such a large part of the lost blood was mobilized from the pulmonary vascular bed in these animals

There was no mobilization of blood from the splanchnic vascular bed in the rats which had received i p injections of pentobarbitone There was on the other hand a marked mobilization of blood from this vascular bed in bled ether anesthetized rats One explanation for this difference may be that in the animals anesthetized with pentobarbitone i p blood could so easily be mobilized from the lungs that there was no need for any large reduction in splanchnic blood volume to take place Muelheims Dellenback and Rawson (1959) did find a big reduction in splanchnic blood volume in bled rats anesthetized with pentobarbitone i p but their measurements were carried out in animals which had lost as much as 75 % of their total blood volume The data presented by Chien and Usami (1969) also show the importance of the splanchnic vascular bed as a blood depot in dogs exposed to a large blood loss By close examination of their results however it appears that small blood losses in their dogs (< 10 %) also caused a reduction in central blood volume without much reduction in splanchnic blood volume It could be that at least under certain conditions the pulmonary vascular bed is the more labile blood depot of the body Small and sudden reductions in circulating blood volume would then primarily be buffered by lung blood volume reductions

However, one cannot exclude the possibility that an i p injection of pentobarbitone may affect the splanchnic vessels and their ability to constrict Rieke and Everett (1957) investigated the effects of i p pentobarbitone anesthesia on the blood content in various tissues in the rat using lightly ether anesthetized animals as controls They found significant alterations in the blood content of several organs upon pentobarbitone anesthesia An increase of the blood volume of the splanchnic organs was particularly marked

In conclusion The present experiments indicate that both the pulmonary and the splanchnic vascular bed can effectively participate in the volume compensation upon a blood loss The relative role of the two vascular areas in this connection is apparently largely depending on the anesthetics used and perhaps also on the degree of bleeding

The authors have been Research Fellows of the Norwegian Research Council for Science and the Humanities Financial support has also been received from the Norwegian Council on Cardiovascular Diseases This support is gratefully acknowledged

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Effect of Brain Lesions and Chlorpromazine on Accumulation and Disappearance of Catecholamines Formed in vivo from ^{14}C-Tyrosine

By

HENRIK NYBÄCK

Received 24 June 1971

Abstract

NYBÄCK, H *Effect of brain lesions and chlorpromazine on accumulation and disappearance of catecholamines formed in vivo from ^{14}C-tyrosine* Acta physiol scand 1972 *84* 54—64

Rats were sul ... thalamic junct ... 6 and 18 h af ... about 50 per cent of that o ... days The content of noradrenaline (NA) tended to be increased 24 h after the lesion but no change was found on the following days The accumulation and disappearance of ^{14}C DA formed from ^{14}C tyrosine in the striatum 12—24 h after the nigral lesion were decreased on the lesion side in comparison with the contralateral side The accumulation and disappearance of ^{14}C NA did not differ between the 2 sides Chlorpromazine accelerated both accumulation and disappearance of ^{14}C DA in the striatum on the side contralateral to the lesion but not in the striatum of the lesion side In animals with a unilateral lesion above the DA pathway, in the posterior ... sides The accumulat ... intact side following ... luenced by the drug ... over in the striatum by activating the nerve impulse flow in the nigro striatal DA pathway

Considerable evidence supports the view that chlorpromazine (CPZ) accelerates synthesis and turnover of catecholamines in brain CPZ increases the brain levels of catecholamine metabolites (Carlsson and Lindqvist 1963 Andén Roos and Werdinius 1964, Laverty and Sharman 1965 Bernheimer and Hornykiewicz 1965, DaPrada and Pletscher 1966) and the rate of disappearance of catecholamines after inhibition of their synthesis with α methyltyrosine (Corrodi Fuxe and Hokfelt 1967, Neff and Costa 1967) Also, the conversion of radioactive tyrosine to catecholamines and the rate of disappearance of the labelled amines are accelerated by CPZ (Burkard

Gey and Pletscher 1967, Nyback, Sedvall and Kopin 1967, Gey and Pletscher 1968, Persson 1969, Nyback and Sedvall 1970) The stimulatory effect of CPZ on the turnover of brain catecholamines has been ascribed to a feed-back activation of catecholamine neurons induced by a blockade of catecholamine receptors (Carlsson and Lindqvist 1963)

In previous studies CPZ was found to accelerate the accumulation and disappearance of dopamine (DA) formed from ^{14}C-tyrosine in the striatum but not in other brain regions (Nybäck and Sedvall 1969, Nybäck 1971) The accumulation and disappearance of ^{14}C noradrenaline (NA) was not significantly affected by the drug in any of the brain regions Since it is known from peripheral tissues that a positive correlation exists between NA turnover and impulse activity in adrenergic nerves (Aluosi and Weiner 1966, Gordon *et al* 1966, Sedvall, Weise and Kopin 1968), it was suggested that CPZ accelerates the impulse flow in the DA neurons which originate in the substantia nigra and terminate in the neostriatum

The present study was undertaken to investigate the importance of the nigrostriatal DA pathway for the effect of CPZ on the metabolism of DA in the striatum Rats were subjected to a unilateral stereotaxic lesion immediately rostral to the substantia nigra in an attempt to interrupt the nerve impulse flow in the DA pathway The effect of CPZ on the accumulation and disappearance of catecholamines formed from ^{14}C tyrosine in the left and right striatum was then studied

Methods

Male Sprague Dawley rats (140—180 g) were anesthetized with pentobarbital sodium (40 mg/kg, i p) and fixed by the head in a stereotaxic apparatus (David Kopf Instr) Lesions were performed mechanically using a stainless steel insect pin (No 000) with a semicircular [illegible] wo types of lesions were [illegible] ig 1 below) where the [illegible] are located (Zeman and [illegible] and another above this [illegible]

At various time intervals following the operation the animals were killed by stunning and decap[illegible] [illegible]

[illegible] NEN Chemicals GmbH 25—50 μCi in 0 5 ml of saline per animal was rapidly injected or infused during 20 min into a tail vein Isotonic saline or CPZ chloride (Hibernal® Leo 10 mg/kg) was injected i p 2 h after ^{14}C-tyrosine injection or 1 h before the start of the ^{14}C tyrosine infusion Animals were killed 2 and 6 h after the ^{14}C tyrosine injection or immediately after the infusion

For evaluation of the results the Student s *t* test for group of paired comparisons were used

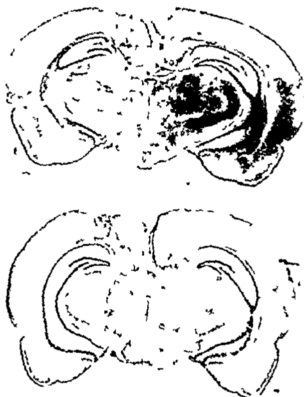


Fig 1 Two representative rat brain slices illustrating the types of lesions used in the text designed as thalamic (above) and nigral (below) lesions 2 weeks after the operations the animals were killed and the brains were removed fixed in formaldehyde for 7 days and stained with cresyl violet

Results

The animals with a nigral lesion but not the animals with a thalamic lesion exhibited a tendency to turn toward the side of the lesion as soon as they had recovered from the anesthesia Apart from this observation which is in accordance with the findings of Ungerstedt (1971) the gross behaviour of the animals was normal

The histological examination of the brains 14 days after the operation revealed some variation in the size and form of the lesion as well as the degree of cortical damage The lesions in Fig 1 are representative for the material

Effects of unilateral brain lesions on catecholamine levels in the striatum and rest of forebrain

At various time intervals after a unilateral brain lesion animals were killed and the levels of endogenous DA and NA were determined in the left and right striatum and rest of forebrain

The level of DA in the striatum ipsilateral to the nigral lesion was increased by about 50 % between 6 and 18 h after the lesion (Fig 2 and 4) At 24 h after the lesion the DA content of the ipsilateral striatum was slightly lower than that of the

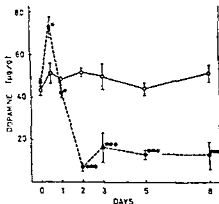


Fig 2

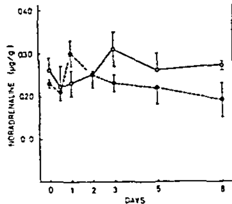


Fig 3

Fig 2 Contents of dopamine in the right (○——○) and left (●----●) striatum at various time intervals after a left nigral lesion Each point is the mean value ±S E for groups of 5—6 animals
* p < 0 05
*** p < 0 001

Fig 3 Contents of noradrenaline in the right (○——○) and left (●----●) striatum at various time intervals after a left nigral lesion Each point is the mean value ±S E for groups of 5—6 animals At none of the time intervals is there a significant difference (p < 0 05) between the right and the left striatum

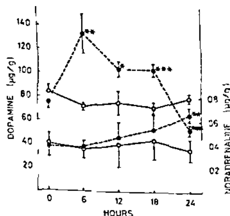


Fig 4

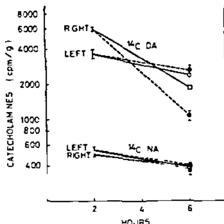


Fig 5

Fig 4 Contents of dopamine (upper two graphs) and noradrenaline (lower two graphs) in the right (○——○) and left (●- -●) striatum at various time intervals after a left nigral lesion Each point is the mean value ±S E for groups of 5—6 animals
* p < 0 05
** p < 0 01
*** p < 0 001

Fig 5 Effect of a left nigral lesion and chlorpromazine treatment on the disappearance of catecholamines formed from ^{14}C-tyrosine in the rat striatum About 18 h after the lesion ^{14}C-tyrosine was injected i v 2 h later one group of animals was killed and the rest were injected with saline (○——○) or chlorpromazine (●- --●) The latter animals were killed 6 h after ^{14}C-tyrosine injection Each point is the mean value ±S E for groups of 5—6 animals

TABLE I Effect of a left posterior thalamic lesion on the catecholamine content (μg/g) of the rat striatum Figures represent mean values ± S E. of 4—7 determinations

Time after lesion	Dopamine		Noradrenaline	
	Right side	Left side	Right side	Left side
6 h	4 78 ± 0 28	4 65 ± 0 17	0 31 ± 0 01	0 32 ± 0 04
3 days	4 64 ± 0 17	4 14 ± 0 38	0 27 ± 0 04	0 33 ± 0 02

TABLE II Effect of a left nigral lesion on the catecholamine content (μg g) of the rat forebrain without striatum Animals were killed 8 days after the lesion Figures represent mean values ± S E of 5—6 determinations

Animals	Dopamine		Noradrenaline	
	Right side	Left side	Right side	Left side
Not lesioned	0 42 ± 0 07	0 46 ± 0 10	0 32 ± 0 03	0 30 ± 0 02
Lesioned	0 40 ± 0 05	0.22 ± 0 04	0 33 ± 0 05	0 29 ± 0 02

contralateral striatum whereas at 48 h and later the DA content was markedly decreased on the lesion side The level of NA was slightly increased 24 h after the lesion (Fig 4) During the following days no significant change in the NA level was found (Fig 3

At 6 h or 3 days after a lesion in the posterior thalamus there were no differences in the amine levels between the right and left striatum (Table I)

Eight days after the nigral lesion the mean DA level of the ipsilateral forebrain was lower than that of the contralateral side (Table II However this difference was not statistically significant p > 0 05)

Effect of unilateral brain lesions on CPZ induced acceleration of ¹⁴C DA accumulation in the striatum

Rats were subjected to a left thalamic or nigral lesion Twelve hours after the operation saline or CPZ was administered Table III One hour later ¹⁴C tyrosine was infused for 20 min Immediately after the infusion the animals were killed and the contents of endogenous and labelled tyrosine and labelled catecholamines were determined in extracts of left and right striatum

The levels of endogenous and labelled tyrosine were about the same in the right and left striatum and in saline and CPZ treated animals (Table III) In the saline treated animals with a nigral lesion the content of labelled DA did not differ significantly between the right and left striatum However if the [illegible] reased DA content (Fig 4) is taken into account it can [illegible] activity of ¹⁴C-DA [illegible]

of 6—7 determinations

Type of lesion	Treatment		Ty (μg g)	^{14}C-Ty spec act (cpm/μg)	^{14}C-DA (cpm/g)	^{14}C-NA (cpm g)
Nigral	Saline	Right side	26 ±1 3	2680 ±267	1920 ±187	336 ±32
		Left side	25 ±0 9	3120 ±359	1440 ±226	323 ±106
Nigral	CPZ	Right side	23 ±1 0	3260 ±275	4800[4] ±521	687[4] ±130
		Left side	23 ±0 8	3420 ±256	1990[1] ±486	277[1] ±36
Thalamic	CPZ	Right side	23 ±0 7	3230 ±211	4030[5] ±378	570[4] ±78
		Left side	20 ±1 3	3450 ±211	3140[1] ±267	393 ±59

[1] Differs from right side ($p < 0.05$) but not from saline group ($p > 0.05$)
[2] Differs from right side ($p < 0.01$) but not from saline group ($p > 0.05$)
[3] Differs from right side ($p < 0.05$) and from saline group ($p < 0.001$)
[4] Differs from saline group ($p < 0.05$)
[5] Differs from saline group ($p < 0.001$)

In animals with a nigral lesion the accumulation of ^{14}C DA in the striatum of the intact side was increased by CPZ (Table III) On the lesion side however CPZ did not affect the accumulation of ^{14}C-DA In contrast in animals with a thalamic lesion CPZ accelerated the accumulation of ^{14}C DA in the striatum of both sides In both nigral and thalamic lesioned animals CPZ accelerated the accumulation of ^{14}C-NA on the right side but not on the left side

Effect of unilateral nigral lesions on CPZ induced acceleration of ^{14}C DA disappearance from the striatum

Rats were subjected to a left nigral lesion About 18 h after the operation ^{14}C tyrosine was injected i v Two h later one group of animals was killed and the remainder were injected with saline or CPZ The latter two groups were killed 6 h after ^{14}C tyrosine injection Contents of endogenous and labelled tyrosine and labelled catecholamines were determined in the left and right striatum

The ^{14}C tyrosine specific activity was found to be the same in right and left striatum and in saline and CPZ treated animals (Table IV) 2 h after ^{14}C tyrosine administration a significantly higher level of ^{14}C-DA was found in the right striatum

[illegible] at 2 and 6 h after precursor administration

Treatment	Time after ^{14}C-tyrosine (hours)		Ty (µg g)	^{14}C-Ty spec act (cpm/µg)	^{14}C-DA	^{14}C-NA
—	2	Right side	13 ±1 8	50 ±6 5	—	—
		Left side	10 ±1.2	66 ±6 9	—	—
Saline	6	Right side	17 ±0 7	19 ±1.2	0 29 ±0 01	0 06 ±0 01
		Left side	14 ±2 1	25 ±4 3	0 11[1] ±0 03	0 09 ±0 02
CPZ	6	Right side	11 ±2 0	26 ±5 2	0 43[2] ±0 02	0 09 −0 02
		Left side	11 ±1 4	24 ±2 7	0 10 0 01	0 07 0 02

[1] Differs from right side ($p < 0.001$)
[2] Differs from saline group ($p < 0.001$)

than in the left (Fig 5 At 6 h, however the level of ^{14}C-DA was higher in the left striatum Therefore the rate constant for ^{14}C-DA disappearance was much lower on the left than on the right side (Table IV) The difference between the right and left striatum is even more pronounced if the decline in specific activity of ^{14}C DA is calculated from endogenous and labelled DA levels in Fig 4 and 5 CPZ accelerated the disappearance of ^{14}C DA on the intact side but not on the side of the lesion No effects of the lesion or CPZ were obtained on ^{14}C NA levels or disappearance constants

Discussion

A pathway of dopamine neurons which originates in the substantia nigra and terminates in the neostriatum has been demonstrated by histochemical (Andén *et al* 1964 and 1965) and biochemical Bertler *et al* 1964, Poirier and Sourkes 1965) methods The time course for the reduction in the number of amine accumulating nerve terminals in the striatum after a lesion of the nigro striatal pathway was studied by Hokfelt and Ungerstedt (1969) using electron and fluorescence microscopy The results of the present investigation on DA levels in the striatum after a nigral lesion (*Fig* 2) are in agreement with the morphological findings of Hokfelt and Ungerstedt (1969) Both studies indicate that the degeneration of DA neurons

is abrupt since a marked reduction in the number of nerve terminals and in the DA content took place between the first and the second day after the lesion

The levels of NA in the striatum were not significantly ($p > 0.05$) reduced by the lesion (Fig 3) although somewhat lower values were obtained on the side of the lesion 3 5 and 8 days after the operation The catecholamine levels in the rest of the forebrain were not significantly reduced either (Table II) These findings indicate that the lesion was fairly well localized to the nigro striatal DA pathway The tendency to decreased DA content of the forebrain on the side of the lesion indicates that the lesion also affected the mesolimbic DA neurons which run medial to the nigro-striatal DA pathway and terminate in the tuberculum olfactorium and other forebrain nuclei (Fuxe 1965 Andén *et al* 1966)

Neither the initial increase nor the decrease in catecholamine levels were present in sham lesioned animals (Nyback and Sedvall 1971) or in animals with a posterior thalamic lesion (Table I) These findings exclude the possibility that the changes in amine levels were due to unspecific effects of the operation

Increased levels of DA in the striatum during the first 24 hours after a nigral lesion (Fig 4) have also been found by Faull and Laverty (1969) and Andén *et al* (1971) The decrease in NA content of skeletal muscles following sympathectomy is also preceded by an initial increase in the transmitter content (Sedvall 1964) Furthermore inhibition of the impulse flow in adrenergic nerves to peripheral organs by decentralization or ganglionic blocking agents gives rise to an increase in the transmitter levels (Hertting Potter and Axelrod 1962 Sedvall 1964) The increase in the catecholamine content after interruption of the impulse flow has been attributed to a diminished release of the transmitter in the presence of an unaltered rate of synthesis (Hertting Potter and Axelrod 1962)

Evidence for an initial slower release of DA from the striatum is furnished by the decreased disappearance of ^{14}C DA on the side of lesion (Table IV, Fig 5) and by the decreased disappearance of endogenous DA after tyrosine hydroxylase inhibition in rats with a nigral lesion (Andén *et al* 1971) However in the present study also the synthesis of ^{14}C DA was decelerated on the side of the lesion (Table III Fig 5) Thus the initial increase in endogenous DA levels seems to be the result of a complex change in the relation between the rates of synthesis and release of DA

The decreased formation of ^{14}C DA from ^{14}C tyrosine in the striatum after an acute lesion (Table III Fig 5) is in analogy with the diminished accumulation of ^{14}C-NA formed from ^{14}C tyrosine in the submaxillary gland after interruption of the adrenergic nerve impulse flow by decentralization (Sedvall Weise and Kopin 1968) Thus a similar relationship between nerve impulse flow and transmitter synthesis seems to exist in peripheral NA neurons and central DA neurons

The rate of catecholamine synthesis is regulated by intraneuronal and interneuronal mechanisms Intraneuronally the level of free amines regulates the rate of tyrosine hydroxylation by end product inhibition (Spector *et al* 1967 Stjarne Lishajko and Roth 1967) The interneuronal regulation operates from catecholamine receptors and modulates the activity of the presynaptic neurons (Carlsson and Lind

qvist 1963) A feed back activation of ^{14}C-DA synthesis and turnover in the striatum (Nybäck and Sedvall 1969, Nybäck 1971) may be brought about by the striato-nigral neurons which have been described by various authors (Voneida 1960, Szabo 1962, Nauta and Mehler 1966, Bedard *et al* 1969)

The previously reported lack of effect of CPZ on ^{14}C-NA metabolism (Nybäck and Sedvall 1969, Nyback 1971) could indicate that the drug does not block NA receptors, or that the normal impulse activity of the NA neurons is too low to be accelerated as a consequence of a receptor blockade The present finding that CPZ increases the accumulation of ^{14}C-NA in the striatum of the unoperated side (Table III) may indicate that following a nigral lesion, the activity of the NA neurons is increased to an extent that a receptor blockade induces a feed back stimulation of the ^{14}C-NA synthesis Evidence for an activation of the NA neurons after the nigral lesion is the fact that in the striatum of the unoperated side the relationship between ^{14}C-NA and ^{14}C-DA was 1/6 (Table III), whereas in unoperated animals it was 1/11 (Nyback and Sedvall 1969) Anden *et al* (1970) have also suggested that blockade of DA neurotransmission causes an activation of brain NA neurons

It was recently found that an acute nigral lesion abolished the CPZ-induced acceleration of ^{14}C-DA formation from ^{14}C tyrosine in the rat striatum (Nybäck and Sedvall 1971) The present results verify and extend this finding In rats with a unilateral nigral lesion CPZ accelerated the formation of ^{14}C-DA only in the striatum contralateral to the lesion whereas in animals with a posterior thalamic lesion the drug accelerated the formation of ^{14}C-DA from the labelled precursor on both sides Also the CPZ induced acceleration of ^{14}C-DA disappearance from the striatum (Nyback 1971 was completely abolished on the side of the nigral lesion

The results thus indicate that the acceleration of DA synthesis and turnover after CPZ treatment is not due to a direct effect of the drug on DA stores in the striatum but requires intact nerve fibres running medial to the crus cerebri at the level of the hypothalamic mesencephalic junction The same conclusion was independently drawn by Anden *et al* (1971) from the finding that CPZ induced acceleration of DA disappearance after tyrosine hydroxylase inhibition was counteracted by a nigral lesion Although NA neurons to the striatum and rest of forebrain appeared unaffected by the nigral lesion (Fig 3 Table II) it is likely that the lesion involved other neurons than DA neurons which might influence DA metabolism in the striatum. However it seems reasonable to assume that the abolition of the CPZ effect is due to transection of nigro striatal DA neurons The present findings thus support the view that CPZ accelerates DA synthesis and turnover in the striatum by activating the nerve impulse flow in the nigrostriatal DA pathway

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General Characteristics of Sympathetic Activity in Human Muscle Nerves

By

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Abstract

Delius, W., K.-E. Hagbarth, A. Hongell and B. G. Wallin. *General characteristics of sympathetic activity in human muscle nerves.* Acta physiol. scand. 1972. 84. 65—81.

Multiunit sympathetic activity was recorded from muscle nerve fascicles in the median or peroneal nerve of resting, relaxed human subjects. The impulses, which were reversibly abolished by a sympathetic ganglion blocking agent, were grouped in the pulse rhythm and series of such pulse rhythmic bursts of impulses regularly appeared during spontaneously occurring blood pressure reductions, whereas temporary blood pressure elevations were associated with neural silence. The findings agree with the notion that the sympathetic muscle nerve activity recorded consists of vasoconstrictor impulses the outflow of which is modulated by a strong ph[illegible] rterial pulse wave to [illegible] 1.4 seconds, dependin[illegible] on a rapid deep bre[illegible] the sympathetic discharges lasting a few seconds.

Pulse and respiratory grouping of discharges in cervical, thoracic and abdominal sympathetic nerves was first reported by Adrian, Bronk and co-workers (Adrian, Bronk and Phillips 1932, Bronk *et al.* 1936). Working on lightly anaesthetized or decerebrate cats and rabbits, they showed that sympathetic cardiac rhythms were dependent upon pulsative inhibitory inflow from baroreceptors and that the sustained rhythmic outflow from the medullary vasomotor centres could be elevated or suppressed in various ways, *e.g.* by pulmonary inflation, asphyxia and changes in blood pressure. They also demonstrated that sympathetic outflow could be modified by hypothalamic stimulation and that sympathetic rhythms could be driven by recurrent stimuli to peripheral sensory nerves. This pioneer work has been followed by several detailed neurographic studies of vasomotor reflexes evoked from various receptors and afferent nerve groups (*e.g.* Gernandt, Liljestrand and Zotterman 1946, Sell, Erdelyi and Schaefer 1958, Weidinger and Leschhorn 1964, Kezdi and Geller 1968, Sato *et al.* 1969) and recent evidence suggests that antagonistic responses

may occur in the sympathetic vasoconstrictor outflow in different nerves (Walther Iriki and Simon 1970 Iriki *et al* 1971)

Until lately, direct recording of sympathetic activity was not possible in man and our present knowledge about human vasomotor tone and reflexes is therefore based upon regional blood flow measurements and estimations of vascular resistance As described by Hagbarth and Vallbo (1968 b), however, a rhythmic neural activity similar to that encountered in visceral sympathetic nerves of animals can be recorded with tungsten microelectrodes inserted into human muscle nerves in situ Since the pulse grouped discharges were found to be transmitted in efferent nerve fibres conducting at about 1 m/s, Hagbarth and Vallbo concluded that they were postganglionic sympathetic and presumably vasoconstrictor signals

The investigation of the sympathetic activity in human muscle nerves has now been extended The present report describes the various resting sympathetic rhythms encountered in muscle nerve recordings and their correlation to spontaneous variations in blood pressure Various ways of driving the sympathetic rhythms are also described and the effect of ganglion blocking agents is shown A separate study deals with the effects of various manoeuvres known to cause reflex changes in the vasoconstrictor tone of skeletal muscles (Delius *et al* 1971 a) In subsequent papers a corresponding analysis is made of the sympathetic outflow in human skin nerves (Hagbarth *et al* 1971 Delius *et al* 1971 b)

Methods

Subjects

The investigations were performed on 7 subjects 4 men and 3 women ranging in age from 24—49 years One of them served as an experimental subject on three occasions All were clinically healthy without signs or symptoms of any neurological or cardiovascular disease

General procedure

The subject lay in the horizontal position The extremity from which the nerve recording was made was placed in a comfortable stable position and the subject was instructed to relax and avoid all active movements The room temperature varied between 20 and 25° C Uncontrolled environmental influences especially sudden noises were avoided The experiments were not performed in a soundproof room however and the subject was fully alert and allowed to talk

Selection of nerve recording site

Human mixed nerve trunks are built up of large numbers of well encapsulated nerve fascicles which are relatively easy to impale After impalement the electrode picks up activity from many large mechano receptive axons within the fascicle Hagbarth *et al* 1970) From the insertion paraesthesias the subject can tell whether a skin or a muscle nerve fascicle has been impaled and his statement can be verified by testing the type and site of peripheral stimuli required to induce afferent signals For muscle nerve fascicles taps on the muscle belly or passive muscle stretches were effective in evoking afferent responses whereas no responses could be induced by skin stimuli Sometimes afferent impulses could be obtained both in response to skin and muscle stimuli Recordings from such mixed fascicles were not included in the present investigation

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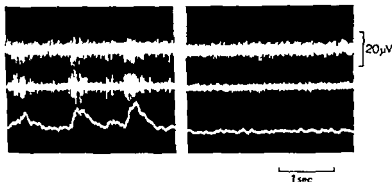


Fig 1 Effect of reducing the bandwidth of the display system on signal to-noise ratio [illegible] Hz [illegible] an integrating circuit [illegible]

Left part of the figure shows sympathetic signals from muscle nerve fascicle Right part shows noise recorded outside the fascicle

Nerve electrodes recording and display systems Nerve recordings were made with insulated [illegible] a diameter of 1—5 μ and a length [illegible]

Hz, a frequency range found to give an optimal signal to-noise ratio for the multiunit sympathetic discharges The time constant of the integrating circuit used to obtain a mean voltage display of the neural activity was 0 11 sec unless otherwise stated In the original nerve recording the amplitude of the bursts seldom exceeded 25 μV whereas the peak to-peak noise level was 7—10 μV Fig 1 illustrates the working principles of our display system with both original and mean voltage neurograms In the figures to be presented in the following only mean voltage neurograms will be shown and in these amplitude calibrations are omitted because of the amplitude distortion inherent in the filtering process

Blood pressure recordings Direct continuous recordings of arterial blood pressure were made through a needle introduced into the left radial artery at the wrist and connected to a pressure transducer EMT 35 and an electromanometer EMT 31 (Elema Schonander Ltd Stockholm)

Respiratory movements were recorded by a strain gauge attached to a rubber band placed around the thorax

ECG was recorded via surface chest electrodes and *the heart rate* was in some cases monitored from the ECG by an instantaneous ratemeter

Painful electrical [illegible] applied through surface electrodes were obtained from a [illegible] (DISA 13GO4) delivering rectangular voltage pulses with a duration of 0 5—1 0 ms and an amplitude of 100—500 V

Results

Search for sympathetic activity in muscle nerves

Pulse grouped sympathetic discharges similar to those described by Hagbarth and Vallbo (1968 b) were found in all subjects but not in all muscle nerve fascicles impaled The signals were sometimes totally absent even though the electrode, as

judged by the afferent multiunit responses to peripheral test stimuli was properly placed within a muscle nerve fascicle. Repeated minor electrode adjustments were often necessary before the characteristic loud speaker sound of pulse grouped discharges indicated that the electrode tip had come close to a bundle of active sympathetic fibres. If care was then taken to avoid further electrode movements the recording conditions could remain stable for several hours.

No serious attempt was made to measure and compare the strength of the sympathetic outflow in the nerves of different persons or in the nerves of the upper or lower extremities. It was noted, however, that in some subjects a great deal of search was required before any pulsative sympathetic signals could be detected and when found they were often of low intensity. On the contrary, in other subjects sympathetic bursts were obtained in most electrode positions and sometimes with a strength great enough to obscure any recording of afferent mechanoreceptor activity. It may be a coincidence but it is worthy of note that the three subjects belonging to the latter category were all above 40 years of age. All illustrations in the present paper were obtained from these subjects.

Evidence for the sympathetic origin of the signals

According to Hagbarth and Vallbo (1968 b) the pulse grouped discharges which persist during arterial occlusion of the limb and distal Lidocaine block of the muscle nerves, are conducted in efferent fibres with a velocity of about 1 m/s. If, as these findings suggest, the signals derive from post ganglionic sympathetic axons one would expect the activity to be reduced or abolished by proximal Lidocaine blockade of the nerve and by ganglion blocking agents.

Such tests have now been performed. In one case the pulse synchronous bursts recorded in the median nerve at the elbow were totally blocked when a dilute solution of Lidocaine was injected around the median nerve in the axillary region. The effectiveness of the sympathetic block was also evidenced by the fact that the subject reported a feeling of warmth in the hand at the same time as the pulsative discharges disappeared.

In the experiment illustrated in Fig. 2 the subject was given a slow i.v. infusion of a ganglion blocking drug (trimethaphan) while continuous recordings of pulse grouped discharges and intra arterial blood pressure were made. During the first 14 min of the infusion when the subject was lying with her feet lowered 20° on a tilted table her systolic blood pressure gradually fell to about 80 mm Hg. To avoid further fall of pressure the table was then tilted to the horizontal position while the infusion continued. The blood pressure fall was accompanied by a gradual reduction of the neural activity which was almost abolished after 16 min. After the infusion was stopped the neural activity had regained its original strength after about 11 min. *During the experiment afferent stretch responses were now* and then elicited by taps on the muscle innervated by the impaled fascicle (not shown in the figure). Contrary to the pulse rhythmic neural activity these afferent mechano-receptor signals were unaffected by the infusion.

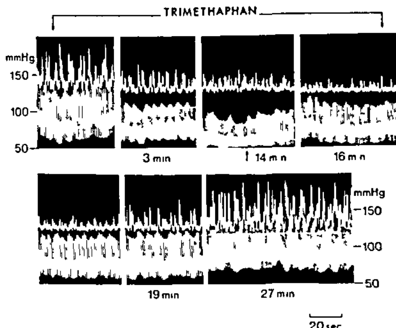


Fig 2 The effect of an intravenous infusion of a ganglion blocking agent (trimethaphan) on the spontaneous sympathetic activity in a peroneal muscle nerve fascicle Arrow below the tracing at 14 min indicates that the position of the subject was changed from 20° head up to horizontal
Upper tracing Integrated sympathetic nerve activity
Lower tracing Arterial blood pressure

The outcome of this test, which has been recently repeated with a similar result in two hypertensive patients, strongly supports the conclusion that the rhythmic neural discharges under consideration derive from postganglionic sympathetic fibres in the muscle nerve fascicles

Spontaneous fluctuations in the pulsatile sympathetic outflow

Fig 3 A and B show various patterns of pulsative sympathetic activity recorded from muscle nerve fascicles in resting, relaxed subjects The pulse grouped multi unit discharges occurred in more or less distinct sequences separated by periods of relative silence During normal quiet breathing this periodicity was sometimes clearly related to the respiratory rhythm (A, left), but on other occasions the discharge sequences lasted for several respiratory cycles and alternated with about equally long silent periods (A, right) The periodicity was sometimes less distinct, as in B left or quite irregular, as in B right

Changes in pulse frequency were always accompanied by similar changes in the sympathetic rhythms It often happened however, that individual pulse grouped discharges fell out of a sequence and occasionally each second discharge fell out so that the nerve rhythm temporarily became subharmonic to the pulse (Fig 3 B, right)

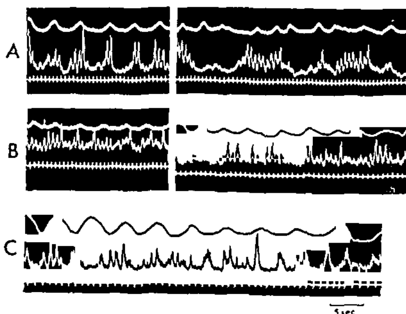


Fig 3 Examples of spontaneous sympathetic activity encountered in muscle nerves under

(inspiration upwards)
nerve activity obtained from median (B right) and

See text.

As shown in Fig 3 A the neural activity did not always cease completely in the pauses between bursts Analysis of the original neurograms verified that this elevation of the basal level was not an artefact caused by the time constant in the mean voltage unit

The tracing in Fig 3 C illustrates an exceptional finding obtained in a peroneal nerve recording from a 42 year old woman In this case the sequences of pulse grouped discharges were intermingled with periodically appearing neural bursts of longer duration not modulated by the pulse rhythm (See Discussion

Blood pressure fluctuations reflected in the sympathetic outflow

In four of the subjects the i a blood pressure was for several hours recorded in parallel with the sympathetic activity in muscle nerve fascicles These recordings revealed that during periods of rest the spontaneous periodic variations in the neural activity were intimately related to similar periodic fluctuations in blood pressure Thus most rhythmic or irregular periodic fluctuations of the pulsative outflow (like those exemplified in Fig 3) were found to be accompanied by similar fluctuations of opposite sign in the blood pressure curve Fig 4 shows typical examples of the relation between a series of irregular arterial blood pressure variations and sympathetic outflow As the systolic pressure fluctuates within the range of 130—160 mm Hg

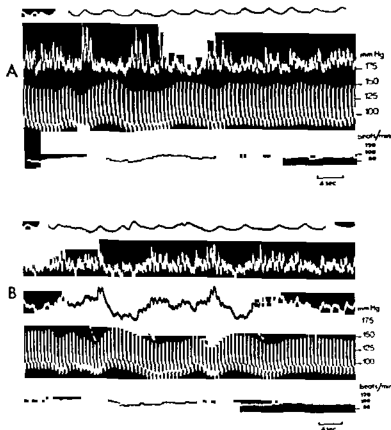


Fig 4 *A* Correlation between intra arterial blood pressure and sympathetic muscle nerve activity recorded in the median nerve Resting subject lying with feet downwards on a slightly tilted table
Upper tracing Respiratory movements (inspiration upwards)
Second tracing Integrated sympathetic muscle nerve activity
Third tracing Intra arterial blood pressure
Fourth tracing Pulse frequency

B Same experiment as in *A* Besides the ordinary mean voltage display of the neural events (*second tracing*) a mean voltage display with a long time constant 1 0 s) was used (*third tracing*) to illustrate how faithfully the spontaneous blood pressure variations were reflected in the neurogram Arrows indicate how periods of increased sympathetic outflow (upper deflections in the neurogram) after a while were succeeded by corresponding upward deflections in the blood pressure curve See text

periods of low pressure are regularly accompanied by outbursts of pulse grouped sympathetic impulses, whereas the crests in the pressure curve correspond to periods of neural silence Fig 4 A illustrates how initial high oscillations in the two curves are gradually damped until the systolic pressure is relatively stabilized at about 150 mm Hg As a rule the systolic and diastolic pressures varied in parallel but when small divergences occurred, as in the latter part of Fig 4 A the periodic changes in

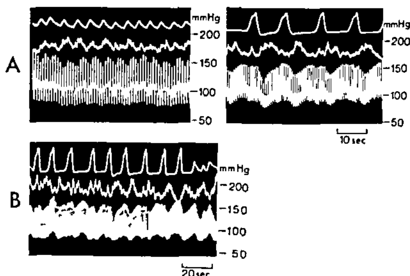


Fig 5 *Various examples of respiratory blood pressure rhythms (lower tracings) reflected in sympathetic outflow recorded from muscle nerve fascicles (middle tracings)* Mean voltage neurograms displayed with long time constant (1 0 s) *Upper tracings* show respiratory movements

A, left Normal spontaneous respiration Median nerve recording

A, right Voluntary deep respiration Peroneal nerve recording

B The same voluntary deep respiration as in *A, right* on another time scale

the neural outflow were better correlated to the systolic than to the diastolic events When the systolic blood pressure changes were rapid it was clearly seen that the corresponding changes in the neural discharges occurred with a time delay of about 1 s This value lies close to the delay estimated for the individual pulsative inhibitions succeeding the heart beats (see below)

To obtain a more satisfactory display of the periodicity in the neural outflow we sometimes used a time constant in the mean voltage unit that was long enough to 'smooth out' the pulse rhythm and bring into better relief the slower changes in neural activity With this type of display it was striking to see how faithfully the pressure changes were reflected in the neurogram (Fig 4 B and 5) It should be noted, however, that the long time constant also caused a small artificial phase lag

Respiratory blood pressure rhythms Periodically the respiratory rhythms were clearly reflected in the blood pressure curve and it was only during such periods that the pulsative neural outflow also exhibited a respiratory periodicity (*cf* Fig 5 A left and right) During normal quiet breathing there was a variable phase relationship between the respiratory movements and the respiratory blood pressure waves but the latter were always coupled to variations of opposite sign in the neurogram Sudden deep inspirations regularly caused a transient rise in blood pressure accompanied by a suppression of the sympathetic outflow (Fig 6 A)

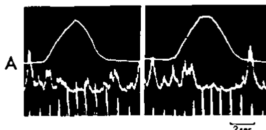




Fig 6 *A* The effect of a sudden deep inspiration on sympathetic muscle nerve activity and [illegible] Same tracings as in *A* with the exception that the lower tracing is ECG (note EMG artefacts from rhythmic muscle contractions in the ECG curve)

It was noticed that blood pressure and neural rhythms driven by respiratory movements of a certain frequency tended to persist during intervening apnoeic periods Furthermore, different respiratory rates were not equally effective in driving such rhythms Deep inspirations occurring at a frequency of about 6/min as in Fig 5 B, appeared to be particularly efficient pacemakers and vasomotor rhythms of this frequency often occurred spontaneously during periods of normal quiet breathing 12/min seemed to be another common frequency for the vasomotor rhythms Fig 6 B illustrates how an initial 6/min rhythm was transformed into an equally regular 12/min rhythm by a series of rapid rhythmic inspirations (combined with fist clenchings) performed at the latter frequency

Quantitative relationship between blood pressure and sympathetic outflow

Although the inverse relationship between the periodic fluctuations in blood pressure and sympathetic outflow was easily recognized it was more difficult to characterize this relationship in quantitative terms As illustrated in Fig 4 A rapid large blood pressure reductions were generally associated with stronger sympathetic bursts than slow blood pressure falls of low amplitude However exceptions were not uncommon and especially in recordings obtained at different time periods (but with unchanged recording conditions during the same experiment) it was easy to find examples where pressure falls of equal speed and magnitude were associated with sympathetic discharges of different strength In the four subjects in whom i a blood pressure

recordings were made the systolic pressure at rest varied from 120—135, 125—160 110—125 and 130—170 mm Hg For several reasons it was difficult to define for any particular subject the exact blood pressure level required to cause a complete suppression of the sympathetic outflow Apart from the technical difficulty of separating very weak bursts from the noise it is likely that the speed of the blood pressure changes has to be considered (Ead, Green and Neil 1952, Gero and Gerova 1962) In addition we noted seemingly spontaneous slow variations in this critical blood pressure level during an experiment This is not surprising as certain manoeuvres involving central commands for circulatory adjustments may alter the strength of the sympathetic outflow independently of the preexisting blood pressure level (Delius *et al* 1971 a)

Periodic fluctuations in sympathetic outflow reflected in the blood pressure

The results described agree with the notion that the sympathetic signals in the muscle nerves originate from vasoconstrictor fibres, the activity of which is modulated by inhibitory inflow from the baroreceptors If resulting resistance variations in the vascular beds of skeletal muscles are of importance for the autoregulation of the blood pressure, one would expect to find a time relationship between the fluctuations in the sympathetic outflow and succeeding blood pressure changes

In Fig 4 most of the periodic fluctuations in the neural outflow were indeed succeeded by corresponding changes in the blood pressure curve However, some of the blood pressure variations were also accompanied by compensatory changes in pulse frequency (Fig 4 A) and thus changes in cardiac output may have contributed in bringing the pressure back towards the previous level Still, in many parts of the recordings (*e g* Fig 4 B) the variations in pulse frequency were negligible and there appeared to be a definite causal relationship between peripheral neural events and succeeding blood pressure waves As indicated by the dotted lines in this figure, suddenly arising high crests in the *neurogram* were often succeeded after 2—3 s by corresponding crests in the blood pressure curve, whereas it often took as long as 4—5 s for slow rises and falls in the neural outflow to be reflected in the systolic blood pressure

Pulsative inhibitions induced by the heart beats

If the pulse synchronous grouping of the sympathetic impulses is caused by pulsative inhibitory inflow from baroreceptors it might be expected, for any particular recording site, that a fixed interval would be found between each systolic pause in the neurogram and a preceding heart beat Fig 7 A left was obtained by superimposing several oscilloscope sweeps triggered by QRS complexes and shows the time correlates between the triggering QRS complexes and the succeeding rhythmic events in a peroneal nerve fascicle When comparing the recordings obtained during a period of low (upper recording) and high pulse frequency (lower recording) the integrated neurograms show that only the systolic pause with a delay of 1 4 s starts at a fixed time after the triggering signal

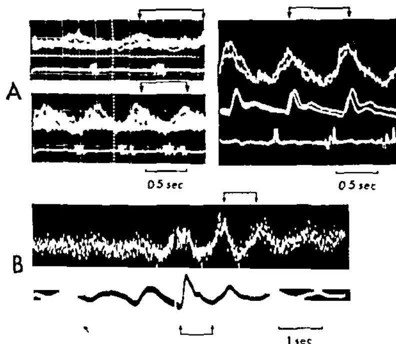


Fig 7. Time correlation between sympathetic muscle nerve activity and pulse frequency (*represented* either by ECG or arterial pulse waves)
A, left C[illegible] recorded in the peroneal nerve at [illegible] 10 superimposed [illegible]
Upper tr[illegible]
Lower tr[illegible]
The sets of arrows indicate the inhibitory phases with a constant latency from the triggering QRS complex
A, right Correlation between ECG and sympathetic activity recorded in the median nerve [illegible]
[illegible] corded in radial artery
[illegible] between sympathetic nerve activity and arterial pulse pressure Not the same subject as in *A* About 600 consecutive oscilloscope sweeps averaged on a CAT computer triggered by QRS complexes (ECG not shown) Trigger point indicated by small break near the middle of the tracings The averaged neurogram preceding the trigger point was obtained by playing the tape backwards
Upper tracing Averaged neurogram (recorded in the peroneal nerve)
Lower tracing Averaged arterial pulse pressure
The two sets of arrows below and above the recording indicate the corresponding systolic phases in blood *pressure and neural activity*

A more accurate way of estimating the delay of the baroreflex is illustrated in Fig 7 B *Sympathetic rhythms recorded from the peroneal nerve at knee level were* fed for one hour (together with radial pulse waves) into a computer (CAT 1000) triggered by the QRS complexes There were several variations in pulse frequency during this long recording and the computer was effective in cancelling out those neural waves and radial pulse beats that were not time-locked to the trigger pulses

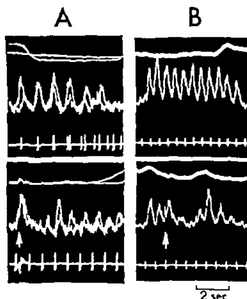


Fig 8 *A* Inhibitory effect of *sudden chest compression* (indicated by arrow in lower half of the figure) on the pulsative sympathetic outflow recorded in a median muscle nerve fascicle Control recordings in upper half of the figure show sequences of pulsative sympathetic outflow prior to the test Two oscilloscope sweeps superimposed in each half of the figure

B Similar effect obtained by *electrical shock* [illegible] lower [illegible] cord [illegible] hetic muscle nerve activity
Lower tracings ECG

(The averaged neural waves preceding the trigger pulses were obtained by playing the tape backwards) The outcome of the test showed that the QRS complexes were optimally correlated to negative deflections in the peroneal nerve recording starting after a delay of 1 2 s

In one subject the QRS complexes were correlated in a similar way to the sympathetic rhythms in both the median and the peroneal nerve The delay of the rhythmic suppressions occurring in the median nerve at the elbow was found to be 0 95 s whereas the corresponding value for the peroneal nerve at the knee was 1 25 s As measured from the upper cervical region the efferent conduction distance to the knee was approximately 0 5 m longer than to the elbow Disregarding possible differences in the intra and extraspinal conduction velocities the values indicate that changes in the sympathetic outflow from the vasomotor centres are transmitted distally with a velocity of 1—2 m/s and that the systolic reductions of this outflow are initiated within about a quarter of a second after the QRS complexes

Externally applied inhibitory stimuli pacemakers for sympathetic rhythms

In several subjects *sudden chest compression* had a definite transient inhibitory effect upon the sympathetic outflow to the muscles (similar to that induced by a sudden deep inspiration see Fig 6 A) In order to see these effects it was necessary to apply the stimuli during periods when the spontaneous slow fluctuations in the sympathetic outflow were relatively stable as in the recordings shown in Fig 8 A Similar transient pauses in the sequences of pulsative sympathetic outflow could be elicited also by *sudden painful stimuli* applied anywhere on the body surface (Fig 8 B) When the stimulus was applied during the rising phase of a pulse grouped discharge in the median nerve the delay of the reflex inhibition was found to be

short enough to cause a falling out of the succeeding pulse discharge Following the pause which did not last for more than 1 or 2 pulse beats there was often a discharge of comparatively high amplitude indicative of a rebound phenomenon As will be shown in a subsequent report the stimuli that induce these reflex pauses in the sympathetic outflow to the muscles have a reciprocal excitatory effect upon the sympathetic outflow to the skin (Hagbarth *et al* 1971)

By trains of recurrent painful stimuli repeated at a constant frequency of about 1/s it was sometimes possible to cause driving of the sympathetic rhythms in the muscle nerves Pulsative inhibitions with succeeding rebounds may well explain this phenomenon which was observed also by Hagbarth and Vallbo (1968 b) who did not note however that each individual stimulus had an initial inhibitory effect

Discussion

Identification of sympathetic signals

Activity in different types of sensory and motor fibres may contribute to the integrated mass activity recorded from human muscle nerve fascicles but for several reasons the sympathetic signals were relatively easily discriminated

a) They were recorded in relaxed subjects without any EMG or other signs of persisting motor unit activity in the limb muscles As previously shown it is mainly during active muscle contractions that the intramuscular stretch receptors exhibit a steady state discharge strong enough to be clearly detectable as a sustained increment of activity in the integrated neurograms (Hagbarth and Vallbo 1968 a) In contrast dynamic multi unit discharges in these afferents are easily evoked by small rapid muscle stretches or light taps on the relaxed muscle bellies Peripheral stimuli of this type were carefully avoided in the present recordings

b) By appearing in irregular sequences and in synchrony with the pulse the sympathetic signals give rise to a very characteristic temporal pattern On rare occasions pulse synchronous afferent signals were also seen They were readily distinguished from sympathetic signals however since they were triggered in a monotonous way by each pulse wave and they could be identified by peripheral test stimuli as coming from vibration sensitive mechanoreceptors

c) The sympathetic bursts had a lower main frequency content than the afferent mechanoreceptive discharges The resulting sound difference could be recognized during the continuous loudspeaker monitoring of the nerve signals

d) Contrary to the intrafascicular mass discharges in large myelinated fibres the sympathetic bursts could be recorded only from certain regions within the fascicles impaled As shown by Gasser (1955) C fibres are not evenly distributed within

the nerves but lie in clusters, many fibres often within the same Schwann's cell It is probably only when the electrode tip lies close to or within such bundles of active sympathetic fibres that the characteristic pulse grouped discharges are seen in the neurogram

Quantitative evaluation of sympathetic outflow

The summation (integration) technique described was used to obtain a relative measure of the number of sympathetic impulses transmitted per unit time by the axons within the recording range of the electrode It should be pointed out, however, that the strength of the neural activity as seen in the integrated neurogram is dependent not only on the number of impulses but also on their amplitude Thus, for any particular electrode site frequency changes in the fibres closest to the recording surface will dominate over similar changes occurring in more distant fibres within the sympathetic bundle Since in multi unit recordings increase of activity in any single unit cannot be distinguished from recruitment of additional units, it follows that random changes in the recruitment order of individual fibres in succeeding bursts may lead to corresponding random variations in the amplitude of the integrated multi unit signals Such random variations in recruitment order may well explain why individual pulsative bursts now and then seemed to fall out of a sequence, whereas others stood out more distinctly than the rest

While consecutive variations in the strength of the sympathetic outflow are relatively easily detected with this recording technique, it is difficult to compare the overall strength of the sympathetic outflow in different muscle nerves or to make interindividual comparisons of the sympathictonus Since the strength of the sympathetic signals varies with the electrode position within the fascicles, recordings from a large number of randomly chosen intrafascicular electrode sites must be made in order to obtain a quantitative measure of the intensity of the overall sympathetic activity Until such systematic samplings have been made the observations mentioned above suggesting differences in strength of the sympathetic activity between individuals of different age must be regarded as preliminary

Baroreflex influence on sympathetic outflow in muscle nerves

All data presented agree with the notion that sympathetic outflow in human muscle nerves during resting conditions is under strong baroreflex influence With the exception of a single case (Fig 3 C) all recordings of sympathetic muscle nerve activity constantly showed pulse synchronous grouping of the impulses In the exceptional case shown in Fig 3 C the pattern of sympathetic activity was similar to that observed in recordings from fascicles containing both skin and muscle nerve fibres (*cf* Hagbarth *et al* 1971) We still do not know whether in this particular case we saw an unusual type of sympathetic muscle nerve activity or whether the recording contained an admixture of sympathetic impulses to the skin

Animal experiments have revealed that pulse synchronous grouping of sympathetic impulses is dependent upon pulsative afferent inhibitory influence from the

carotid sinus and aortic arch pressure receptors, (*e g* Bronk *et al* 1936, Gernandt *et al* 1946, Weidinger and Leschhorn 1964, Green and Heffron 1968) and it seems likely that the mechanism is similar also in man Furthermore the present recordings showed a persistent intimate relationship between fluctuations in systolic blood pressure and sympathetic outflow, a fall in pressure being followed with great regularity by an increase in sympathetic activity It is well known from animal experiments that at low pressures the baroreceptors respond mainly in a dynamic fashion during each systole, whereas at higher pressures a static baroreceptor discharge also appears (Bronk and Stella 1932, McCubbin, Green and Page 1956) This may well explain why during periods of low pressure the sympathetic outflow was high and pulsative whereas during periods of high pressure the inhibitions often became sustained and potent enough to eliminate all signs of sympathetic activity in the neurogram

As a consequence of the relatively low signal to noise ratio it was not always possible to tell whether the sympathetic signals ceased completely during periods of high blood pressure or whether they became so weak that they vanished in the noise Sometimes during periods of low blood pressure, however, it was quite evident that a weak activity persisted also in between succeeding bursts (as in Fig 3 A), indicating that the phasic pulsative inhibitions were not then sufficient to cause complete pauses in the sympathetic outflow On rare occasions the action potentials of a few sympathetic units appeared rather distinctly in the neurogram They fired asynchronously together with other units during the bursts but remained silent in the intervening periods

Simultaneous recordings have not been made from different muscle nerve fascicles or from different nerves in the upper and lower extremities However, the same intimate coupling between systemic blood pressure variations and sympathetic neural events were seen whether the signals were recorded from upper or lower extremity nerves Thus, it is likely, with allowance for phase lags due to different conduction distances, that the spontaneous variations in the sympathetic outflow occur in synchrony in the various muscle nerves The pulsative sympathetic bursts released during periods of low pressure are apparently transmitted distally at a speed of about 1—2 m/s This implies that an impulse volley destined for distal muscle groups may not yet have reached its destination when a succeeding volley to proximal muscles has already impinged upon the effectors

Vasoconstrictor signals

It seems safe to conclude that the baroreflex controlled sympathetic signals originate from those fibres which regulate the vasoconstrictor tone in the skeletal muscles presumably by acting primarily on the precapillary resistance vessels (Cobbold *et al* 1963) If this conclusion is correct one should expect the periodic fluctuations in sympathetic outflow to be followed by corresponding vasomotor changes in the musculature Such spontaneous changes in vasomotor tone sometimes coupled to the resting respiratory rhythm (2nd order waves), but often exhibiting slower

rhythms coupled to spontaneous blood pressure waves (3rd order waves) have previously been described (Koepchen *et al* 1968) There is little reason to doubt that such spontaneous vasomotor waves of the 2nd and 3rd order occur as a consequence of rhythmic fluctuations in sympathetic outflow similar to those described in the present study Single rather than multi unit recordings are required to tell whether the vasoconstrictor signals in the muscle nerves originate from sympathetic efferent neurons which are all equally sensitive to phasic as to static inhibitory inflow from the baroreceptors

Sympathetic fibres causing active vasodilatation in skeletal muscles have been demonstrated in certain animal species (Bulbring and Burn 1935, Eliasson *et al* 1951 Bolme Uvnas and Wright 1969) but there is as yet no definite proof that such fibres exist in man (Bolme and Fuxe 1969) If they exist, one might expect this emergency system to be inactive in resting relaxed subjects and since they are not supposed to be under baroreflex control (Uvnas 1960) they can hardly be the generators of the sympathetic signals described

It can certainly not be excluded however, that besides the baroreflex controlled sympathetic impulses there are other efferent autonomic signals in the muscle nerves which are too weak to be detected with the present recording technique

Genesis of the vasomotor rhythms

It has been suggested that spontaneous vasomotor rhythms may be due to oscillations in the negative feed back circuits engaged in the autoregulation of the blood pressure but rhythms inherent in the vasomotor centres have also been proposed (Koepchen 1962 Weidinger and Leschhorn 1964) In the present study we noted during rest no rhythmic fluctuations in the vasoconstrictor outflow that occurred independently of preceding blood pressure waves Furthermore periods of strong sympathetic outflow were usually succeeded by blood pressure elevations which in turn were followed by suppression of the outflow The phase lag between a period of enhanced neural activity and a succeeding blood pressure elevation varied with the frequency of the prevailing rhythms but time delays of about 4 s were not uncommon With a 1 s delay for the baroreflex this gives a revolution period of about 5 s which would correspond to an oscillation frequency of about 6/min *i e* one of the sympathetic rhythms most commonly observed In conclusion although inherent oscillatory tendencies in the vasomotor centres may well exist our findings as regards the vasomotor rhythms are explicable in terms of oscillations in negative feed back circuits

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Manoeuvres Affecting Sympathetic Outflow in Human Muscle Nerves

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Abstract

Delius, W, K-E Hagbarth, A Hongell and B G Wallin *Manoeuvres affecting sympathetic outflow in human muscle nerves* Acta physiol scand 1972 84 82—94

Multi unit sympathetic activity was recorded in human muscle nerves together with measurements of intra arterial blood pressure and forearm or calf blood flow, during manoeuvres causing circulatory adjustments Manoeuvres causing an increased vascular resistance in the forearm or calf were regularly associated with an increase in sympathetic outflow, proving that the neural activity was dominated by vasoconstrictor impulses The inverse changes of blood pressure and sympathetic activity observed during Valsalva's manoeuvre and mental stress are explicable in terms of baroreflex control of the neural outflow However, during muscle work and hyperventilation the relation between blood pressure and sympathetic activity was more complex indicating that the baroreflex influence was superimposed or modified by other regulatory mechanisms Some manoeuvres known to affect sympathetic outflow in skin nerves caused no appreciable change in sympathetic muscle nerve activity

The sympathetic neural activity recorded from muscle nerves in awake, relaxed human subjects consists of bursts of impulses occurring pulse synchronously in short, rather irregular sequences separated by periods of relative silence (Hagbarth and Vallbo 1968) In a preceding paper it was shown that the occurrence of the rhythmic bursts was intimately related to spontaneous variations in the systolic blood pressure, with the burst sequences appearing during temporary blood pressure reductions (Delius et al 1971 a It was suggested that the impulses caused vasoconstriction and both the pulse synchrony and the close relationship to the spontaneous blood pressure variations were interpreted as expressions of influence of baroreflex mechanisms on the sympathetic activity

From circulatory studies on man several types of manoeuvres, such as changes of body position, Valsalva's manoeuvre muscle work etc are known to elicit typical changes in muscle vascular resistance, partly as a result of an altered sympathetic nerve activity These well documented vascular resistance reactions have formed background data for the present experiments in which sympathetic activity in muscle

nerves has been recorded during several such manoeuvres The results lend further support to the hypothesis concerning the vasoconstrictive nature and the baroreflex control of sympathetic muscle nerve activity In some of the manoeuvres, however, other governing mechanisms were involved, as evidenced by the fact that the strength of the sympathetic outflow changed without preceding alterations of the blood pressure

Methods

Subjects This paper is based on recordings of sympathetic activity from 23 muscle nerve fascicles in the median nerve at the elbow or the peroneal nerve at the fibular head The [illegible] sing the same 7 subjects as in a previous [illegible]

[illegible] well as techniques used for recording of [illegible] *e, respiratory movements and ECG* have [illegible] a) [illegible] 5 subjects by venous occlusion plethysmography, using an air filled rubber cuff and expressed in terms of ml 100 ml^{-1} min^{-1} The forearm or calf *plethysmograph enclosed* a 5 cm long segment of the *muscular part* of the extremity A proximal venous occlusion cuff was applied on the upper arm or on the thigh and a distal occlusion cuff 2—3 cm below the plethysmograph on the forearm or calf For blood flow measurements the proximal cuff was inflated to 60 mm Hg and the distal cuff was inflated simultaneously to 85—90 mm Hg As a rule the blood pressure and flow were measured in different extremities In one case, however forearm blood flow measurements were performed while the arterial blood pressure was being recorded in the radial artery of the same arm [illegible]

[illegible] test situation for 10—15 min before any manoeuvre was performed Due to technical difficulties it was not possible to perform all manoeuvres in all subjects In four subjects the intra arterial blood pressure was recorded throughout the experimental session Except in tests including movements of the extremities venous occlusion plethysmography was performed on the extremity contralateral to that in which the nerve recording was made The following manoeuvres were used

a) *Passive changes of body position* A head up position was achieved by tilting the experimental table 30—45° Passive raising of the legs was used to drain blood from the legs

b) *Valsalva's manoeuvre* After an inspiration of moderate depth the subject made a vigorous expiratory effort against the closed glottis for at least 10—15 s The expiratory effort was not standardized to a given pressure

c) *Muscle work* was performed either as strong isometric hand contractions around a dynamometer or as an isometric adduction of the legs against the sides of the foot plate

d) *Emotional stress* was achieved by unexpectedly asking the subject to perform mental arithmetic which usually consisted of either multiplication of 2 figures numbers or continued subtractions

e) *Hyperventilation* was performed in atmoshperic air and the subject was only instructed to take rapid deep breaths

f) *Body cooling* was achieved by removing the covering blanket and exposing the subject to cooled air At the same time an ice bag was placed on the trunk

g) *Cigarette smoking*

Sources of error in the nerve recording Sometimes when a manoeuvre was performed vigorously small alterations of the electrode position could occur and lead to changes in the

recording conditions simulating a real change in sympathetic activity Great care was taken to avoid such artefacts and whenever there was reason to suspect that the manoeuvre had changed the position of the electrode tip the result was discarded

Although the subjects were instructed to relax the extremity from which the nerve recording was made, muscle contractions sometimes occurred during the manoeuvre, causing EMG artefacts in the neurogram By their different frequency content and lack of pulse rhythmicity such artefacts were usually easy to distinguish from the nerve signals

Results

Before going into the results it should be emphasized that it was not the aim of the present experiments to quantify the nerve response during the manoeuvres but rather to use the manoeuvres as tools for analysing different regulatory mechanisms involved in the control of the sympathetic outflow During a manoeuvre the sympathetic responses did indeed vary markedly in strength from one trial to another or from one recording site to the next Several factors such as differences in the location of the electrodes within the fascicles, differences in effort involved during the manoeuvres differences in age between subjects, etc could contribute to these variations However, contrary to the differences in strength, no variability was observed with respect to the type of the responses, either between the individuals or between different muscle nerves

Passive changes of body position

a) *Head up tilting* When the experimental table was tilted, raising the subject from a horizontal to a more upright position, an increase in sympathetic activity was

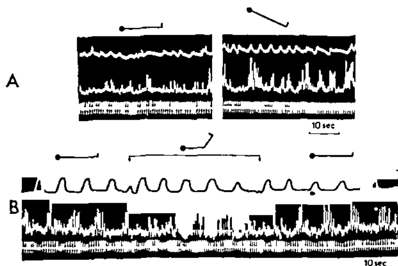


Fig 1 Effect of passive changes in body position on sympathetic muscle nerve activity

A Effect of tilting the experimental table 30° from the horizontal to a head up position. Peroneal nerve recording

B Effect of passive raising of both legs approximately 50° Nerve activity recorded in the right median nerve Note the simultaneous regular deep respiration

Upper tracings Respiratory movements (inspiration upwards

Middle tracings Integrated sympathetic muscle nerve activity

Lower tracings ECG

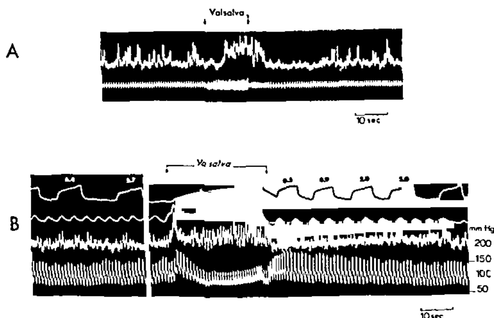


Fig 2 Two examples of the effect of Valsalva's manoeuvre on sympathetic muscle nerve activity

A

Upper tracing Integrated sympathetic activity from a peroneal nerve fascicle
Lower tracing ECG Note EMG artefact during the manoeuvre

B

Upper tracing Forearm blood flow measured by venous occlusion plethysmography The numerical values are expressed in ml 100 ml^{-1} min^{-1}
Second tracing Respiratory movements (inspiration upwards)
Third tracing Integrated sympathetic nerve activity from a median nerve fascicle
Lower tracing Intra arterial blood pressure

commonly observed in muscle nerves of both leg and arm Fig 1 A shows an example of such a reaction which is taken from a recording in the right peroneal nerve when the subject was tilted about 30° from the horizontal position

b) *Raising of the legs* When the legs of a recumbent subject are passively raised the forearm blood flow usually increases (Roddie and Shepherd 1956) This manoeuvre was performed 3 times in 2 subjects when recording from the median nerve In one case no effect on the neural activity or vascular resistance was noted whereas in the other subject a decrease in the sympathetic outflow was observed on two occasions This latter subject was a 44 year old man with quite a pronounced resting activity During a series of deep inspirations the pulse synchronous bursts were grouped very regularly in the respiratory rhythm and it was easy to distinguish a clear reduction in the intensity of the bursts confined to the time period when the legs were raised (Fig 1 B)

Valsalva's manoeuvre

This test was always accompanied by highly characteristic changes of the sympathetic activity and in all 8 expts. where simultaneous nerve and i.a. blood pressure recordings were made an intimate relationship was observed between blood pressure changes and neural events.

Blood pressure variations reflected in sympathetic outflow. With regard to the systolic blood pressure level the effect of Valsalva's manoeuvre can be divided into three parts (Fig. 2 B): 1) An initial transient increase above the resting level caused by the abrupt increase in intrathoracic pressure, 2) A period of decreased systolic pressure lasting until a few seconds after the end of the manoeuvre and caused by a reduction of the effective filling pressure and stroke output of the heart, 3) A fairly long lasting period of increased pressure after the manoeuvre during which the final circulatory readjustments take place. In Fig. 2 A and B it is easy to distinguish changes in sympathetic nerve activity corresponding to these three phases of systolic pressure changes. Both during the initial blood pressure increase as well as during the long lasting overshoot after the manoeuvre the sympathetic discharges were inhibited. (The initial inhibition is best seen in Fig. 2 A; in Fig. 2 B that part of the curve is obscured by a large EMG artefact.) In contrast during the period of decreased systolic pressure the nerve recordings show an intense sympathetic activity. The intimate relationship between blood pressure changes and neural events is illustrated by the fact that during the whole sequence shown in Fig. 2 B the *sympathetic bursts appeared only when the systolic blood pressure fell below approximately 150 mm Hg.* A delay of about 1 second was often observed between the initial rise in blood pressure and the accompanying temporary inhibition of the neural outflow. This value lies close to the delay estimated for the pulsative neural inhibitions succeeding the heart beats (Delius *et al.* 1971 a). Even when the systolic blood pressure fell during the manoeuvre to about 100 mm Hg the phasic neural inhibitions accompanying the heart beats were in some cases still strong enough to cause pulsative interruptions of the sympathetic outflow (Fig. 2 B). In other instances however these pulsative inhibitions became less marked during the manoeuvre so that the enhanced sympathetic activity appeared as a more sustained discharge (Fig. 2 A).

Haemodynamic effects of sympathetic activity. Fig. 2 B shows that after the initial increase the systolic blood pressure fell rather rapidly to about 120 mm Hg where it levelled off and even increased slightly towards the end of the manoeuvre. The levelling off occurred 3.0–3.7 s after the first sympathetic burst had reappeared in the neurogram (mean [illegible] 7 expts.), the obvious interpretation being that the bursts contain vasoconstrictive impulses causing an observable blood pressure change after the delay mentioned.

Forearm or calf blood flow measurements showed either no significant change (as in Fig. 2 B) or a transient blood flow decrease after Valsalva's manoeuvre. The vascular resistance, however, increased (range 12–18 %) in all 5 expts. in which

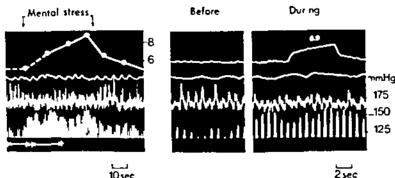


Fig 3 The effect of mental arithmetic on sympathetic muscle nerve activity, systolic blood pressure and forearm blood flow Nerve activity recorded in the right median nerve The two detail recordings to the right are on an expanded time scale and were taken before and during the manoeuvre as indicated by horizontal bars in the lower part of the review (left) recording Same tracings as in Fig 2 B The numerical values for forearm blood flow are expressed in ml 100 ml⁻¹ min⁻¹ The first point in the blood flow recording represents the mean of 3 measurements made before the test

successful simultaneous recordings of neural activity blood pressure and flow were made It should be noted that the increase in vascular resistance which subsided in about 20 seconds, occurred during the periods of neural inhibition following the manoeuvre

Mental stress

Mental arithmetic was performed 6 times by 4 subjects Fig 3 shows a recording from such an experiment where the period of mental strain caused a blood pressure increase At the same time a reduction in strength of the sympathetic activity and a decrease in the forearm vascular resistance were recorded Similar results were obtained in 5 of the 6 expts and in these the systolic blood pressure increased by 20—40 mm Hg In the remaining case no significant change in sympathetic activity was observed Thus the blood pressure elevations caused by mental stress as well as those seen during Valsalva's manoeuvre were accompanied by inverse changes in sympathetic vasoconstrictor outflow to the muscles The blood pressure elevations were never preceded or accompanied by enhancements of the pulsative sympathetic outflow

Muscle work

Periods of fairly intense isometric muscle contractions were performed on 9 occasions in 6 subjects while recording from a nerve supplying a muscle not engaged in the work 7 times a slight to i

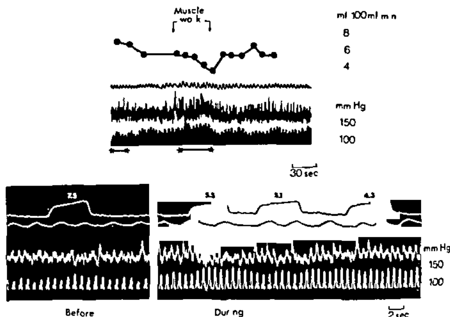


Fig 4 Effect of muscle work (isometric leg adduction) on sympathetic muscle nerve activity recorded in the right median nerve Note simultaneous increase of nerve activity and systolic blood pressure during the work period
The details in the lower part of the figure were taken during periods indicated by horizontal bars in the upper recording Same tracings as in Fig 2 B and 3

bursts was noted in one case the increase in activity during the manoeuvre was negligible but after the manoeuvre a clear reduction of activity was seen and in the remaining case no change was observed In contrast to the inverse relation between blood pressure and sympathetic muscle nerve activity seen during Valsalva's manoeuvre and mental stress the increase of nerve activity during muscle work occurred in parallel with an increase in blood pressure with the neural change often starting a few seconds before the blood pressure rise Fig 4 shows a representative example from the 4 expts where measurements of the i a blood pressure and blood flow were made simultaneously with the muscle contraction As summarized in the upper part of the figure during the muscle contraction both the sympathetic activity and systolic blood pressure increased and the forearm blood flow fell concomitantly As shown by the detail in the lower right part of the figure the normal rhythmical inverse relationship between the sympathetic bursts and the variations in systolic blood pressure could still be observed Consequently although the general increase of sympathetic activity during the manoeuvre cannot be explained as a baroreflex response, the recording suggests that the baroreflex mechanism nevertheless operated during the manoeuvre

Fig 5 Effect of voluntary hyperventilation on sympathetic muscle nerve activity, systolic blood pressure and forearm blood flow Nerve activity recorded in the right median nerve
Same tracings and symbols as in Fig 2 B, 3 and 4

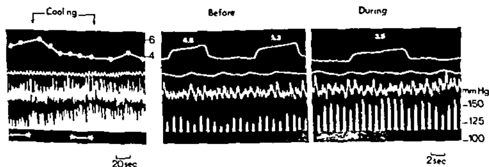


Fig 6 Effect of cooling of the body surface on sympathetic muscle nerve activity (recorded in the right median nerve) systolic blood pressure and forearm blood flow
Same tracings and symbols as in Fig 2 B 3 4 and 5 Blood flow expressed in ml 100 ml ^{1} min ^{1}

Hyperventilation

The hyperventilation test was performed in 2 subjects and on both occasions the manoeuvre resulted in a reduction of the pulsative neural outflow and a concomitant moderate fall in blood pressure In the experiment illustrated in Fig 5 the sympathetic bursts were not very pronounced before the manoeuvre but as shown

both by the summary (upper part of the figure) and the details (lower part) there was a clear reduction of both the amplitude and number of sympathetic bursts during the hyperventilation In parallel with this a decrease in systolic blood pressure and an increase in forearm blood flow were registered Although the sympathetic bursts were difficult to distinguish during the hyperventilation, the small bursts that did appear were usually seen during the minor transient reductions in systolic blood pressure again suggesting that the baroreflex was still operating during the manoeuvre

Body cooling and cigarette smoking

Body cooling was performed twice when recording from a muscle nerve As exemplified in Fig 6 there was hardly any significant change in sympathetic activity although there was a clear increase in blood pressure The slight decrease in blood flow seen in the figure was probably due to constriction in skin vessels The effect of *smoking a cigarette* was also tested twice and the results were similar to those obtained during body cooling *i e* a slow increase in blood pressure but no significant effect on the sympathetic discharges or vascular resistance in muscle vessels As will be shown in a separate report (Delius *et al* 1971 b) these two manoeuvres are efficient in enhancing sympathetic outflow to the skin and skin vasoconstriction may well have contributed to the blood pressure elevations described

Discussion

Evidence for vasoconstrictive nature of the sympathetic outflow

Hagbarth and Vallbo (1968) suggested that the pulse synchronous bursts of sympathetic impulses recorded in human muscle nerves cause vasoconstriction in skeletal muscle The finding that during rest an increase in sympathetic activity regularly was followed by an increase in blood pressure agrees with this interpretation (Delius *et al* 1971 a) In the present investigation head up tilting Valsalva's manoeuvre and muscle work were found to cause an increase in sympathetic activity whereas passive elevation of the legs mental stress and hyperventilation in most cases resulted in a decreased sympathetic outflow As the first three manoeuvres are known to be associated with vasoconstriction and the last three with vasodilatation (for references see below) the vasoconstrictive nature of the sympathetic action potentials grouped in the pulse rhythm seems firmly established

Changes in sympathetic outflow explicable in terms of baroreflex effects

Both the pulse synchronous grouping of impulses and the inverse relationship between the strength of the sympathetic activity and the systolic blood pressure level strongly suggest that sympathetic outflow to skeletal muscle is modulated by arterial baroreflex mechanisms (Hagbarth and Vallbo 1968 Delius *et al* 1971 a) This agrees with earlier results showing that the vascular resistance in human skeletal muscle

is affected by changes in arterial baroreceptor activity (Carlsten *et al* 1958, Bevegård and Shepherd 1966, Epstein *et al* 1969) In the present investigation changes in body position, Valsalva's manoeuvre and mental stress elicited changes in sympathetic outflow in muscle nerves which at least partly are explicable in terms of baroreflex effects

a) *Passive changes of body position* are known to cause reflex changes in muscle blood flow Tilting into the head up position causes a blood flow reduction in the forearms and legs (Florkin, Edwards and Dill 1930, Brigden, Howarth and Sharpey Schafer 1950, Pentecost, Irving and Shillingford 1963), while tilting into a head-down position or passive raising of the legs is associated with an increase in the forearm blood flow (Roddie and Shepherd 1956, Pentecost *et al* 1963 Graf 1965) In agreement with these results we found that a postural change from the horizontal to a more vertical position gave an increase in sympathetic muscle nerve activity, whereas passive raising of the legs reduced the sympathetic outflow to skeletal muscles in the arm Arterial baroreceptors may not be solely responsible for this effect, but as emphasized by Roddie, Shepherd and Whelan (1957) the reflex response to passive raising of the legs may also be initiated by receptors in the low pressure system

b) *Valsalva's manoeuvre* It has been suggested that the rapid fall in blood pressure during Valsalva's manoeuvre causes a peripheral vasoconstriction which prevents a further pressure reduction (Sarnoff, Hardenbergh and Whittenberger 1948, Sharpey-Schafer 1953) This hypothesis was confirmed by the present results, which showed an intense increase in sympathetic activity in muscle nerves during the manoeuvre Furthermore, the temporary inhibition of the sympathetic discharges during the transient blood pressure elevations at the beginning and after the end of the manoeuvre are in full agreement with a baroreflex regulation of sympathetic outflow Several workers have previously found a reduced forearm blood flow during the period of increased blood pressure following Valsalva's manoeuvre (Sharpey Shafer 1953, Roddie, Shepherd and Whelan 1958 Graf 1965) In the present experiments the sympathetic discharges always ended at the very beginning of the blood pressure overshoot, i e within a few seconds after the end of Valsalva s manoeuvre Thus the low forearm blood flow cannot be due to a continuing vasoconstrictive neural outflow but must rather be caused by the inertia of the muscular resistance vessels when relaxing after an intense contraction Possibly a local myogenic reaction to the increasing blood pressure may also constribute to the high vascular resistance (Folkow 1964)

Although sympathetic outflow in muscle nerves during Valsalva s manoeuvre is explicable in terms of baroreceptor control other influences cannot be totally excluded As the manoeuvre is associated with contractions in thoracic and abdominal muscles effects similar to those seen during muscle work may also be present However, no convincing evidence supporting this possibility has yet been found

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Comparison with sympathetic outflow in skin nerves

Under resting conditions the pattern of the sympathetic activity recorded from skin nerves differs markedly from that found in muscle nerves (Hagbarth *et al* 1971) In addition we have found that the manoeuvres used in this investigation usually produce quite different sympathetic responses in the two nerve types A detailed comparison will be made in a forthcoming paper (Delius *et al* 1971 b), and it may suffice to mention here that the differences observed agree with earlier results from blood flow measurements suggesting a selective neural control of skin and skeletal muscle vessels

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From the Department of Physiology, University of Goteborg, Sweden

Convergence in the Reciprocal Ia Inhibitory Pathway of Excitation from Descending Pathways and Inhibition from Motor Axon Collaterals

By

H Hultborn and M Udo

Received 1 July 1971

Abstract

Hultborn, H and M Udo *Convergence in the reciprocal Ia inhibitory pathway of excitation from descending pathways and inhibition from motor axon collaterals* Acta physiol scand 1972 *84* 95—108

With intracellular recording from motoneurones and utilizing the technique of spatial facilitation it has been investigated if those Ia inhibitory interneurones which receive excitation from supraspinal centres also can be inhibited from recurrent motor axon collaterals For each motoneurone the stimulation strengths were chosen so that separate stimulation of either the evoke any IPSP while a combined stimulation at n preceded by stimulation of ventral roots this test found in about 100 motoneurones belonging to dif appeared after facilitation from vestibulo-, rubro- or cortico-spinal tracts

It is thus concluded that a convergence of supraspinal excitation and recurrent inhibition occurs on the same Ia inhibitory interneurones

The disynaptic reciprocal Ia inhibition from muscle spindles to antagonistic motoneurones is facilitated at an interneuronal level from several descending pathways The first example was the excitatory control of the Ia inhibitory interneurones by volleys in the corticospinal tract (Lundberg and Voorhoeve 1962) Later it has been shown that facilitation in this pathway is produced also from the rubrospinal tract (Hongo, Jankowska and Lundberg 1969 b) and the vestibulospinal tract (Grillner Hongo and Lund 1966) A weak facilitatory effect was at a few occasions seen also on stimulation of the medial longitudinal fascicle (Grillner Hongo and Lund 1968 *cf* also 1971)

Recent investigations have shown that antidromic volleys in ventral roots cause a depression of the transmission in the Ia inhibitory pathway Hultborn Jankowska and Lindstrom 1971 a) This depression is by all criteria caused by postsynaptic inhibition of the interposed interneurones which is evoked through recurrent motor

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trol Small electrolytic lesions were made in the stimulation sites at the end of the experiment and the electrode locations were checked histologically

For stimulation single or trains (250—400/s) of 0 2 ms negative square pulses were delivered to the stimulating electrode against an indifferent electrode in the neck muscles The current intensity was always measured and did not exceed 100 μA

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(1957 a) The conditioning shock stimulation of the ventral roots was supramaximal for α fibres and preceded the test response with 6—20 ms The stimulus repetition rate was about 1/s

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Results

The present study concerning the convergence on interneurones mediating the reciprocal Ia inhibition is based exclusively on studies of their synaptic actions in motoneurones Spatial facilitation has been used to reveal an excitatory convergence onto these interneurones from Ia afferents and various descending pathways Provided that the stimulus strengths of a nerve (known to evoke reciprocal Ia inhibition in the recorded motoneurone) and a supraspinal structure is subthreshold for eliciting spikes in the interneurones, their separate stimulation will not evoke any IPSP in the motoneurone Conjoined stimulation of both (at proper intervals) may fire a proportion of the interneurones receiving excitatory action from both these pathways and the resulting IPSP recorded in the motoneurone is thus a manifestation of this convergence

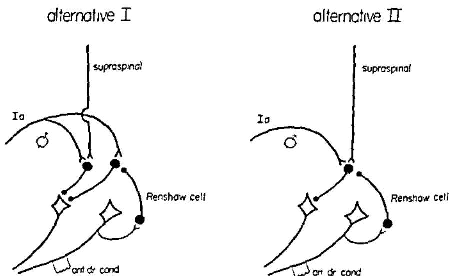
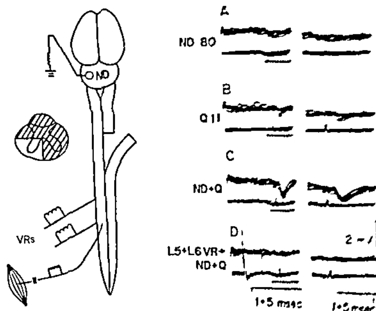


Fig 1 Schematic representation of 2 possible alternatives for the organization of descending excitation and recurrent inhibition (via motor axon collaterals) on the Ia inhibitory interneurones Notice that the descending excitation of the Ia inhibitory interneurones may be either monosynaptic (vestibulospinal tract) or polysynaptic (rubrospinal and corticospinal tracts) Further explanation in text

If there are two subgroups of Ia inhibitory interneurones, one receiving excitation by volleys in a descending pathway and the other inhibition by volleys in motor axon collaterals (alternative I in Fig 1) a preceding conditioning stimulation of ventral roots will not decrease the IPSP resulting from a spatial summation between Ia and descending excitatory actions If, on the other hand, the same population of Ia inhibitory interneurones receive excitation by volleys in a descending pathway as well as recurrent inhibition (alternative II in Fig 1) the IPSP evoked by the spatial facilitation will be decreased or abolished by a conditioning ventral root stimulation

It was often impossible to avoid that separate stimulation of either the peripheral nerve or the supraspinal structure in themselves evoked a small IPSP in the motoneurone Nevertheless it was usually possible to show the convergence of descending excitation and recurrent inhibition on interneurones in the Ia inhibitory pathway This conclusion required the demonstration that the recurrent depression included also the part of the IPSP caused by the spatial facilitation as must be the case when the decrease of the facilitated IPSP by the antidromic conditioning volley was larger than the algebraic sum of the IPSPs evoked by separate stimulation of Ia afferents and descending fibers The significance of this indirect technique for investigation of convergence on the Ia inhibitory interneurones will be further considered in the Discussion

1 *The vestibulospinal tract* Facilitation of Ia IPSPs by volleys in the vestibulospinal tract is seen in motoneurones supplying knee hip and some ankle flexors as well as some hip extensors (Grillner, Hongo and Lund 1966 and to be published)



[illegible] respectively The expanded part of the slow records is indicated [illegible] ... is shown in the left part of the figure (further description under Methods) [illegible]

In the present investigation PBSt and ABSm motoneurones were sampled

Fig 2 demonstrates a convergence of vestibulospinal excitation and recurrent inhibition on interneurones in the Ia inhibitory pathway to a [illegible] motoneurone Separate stimulation of Deiters' nucleus (A) or the Q nerve (B) evoked hardly any IPSP but when stimulated together (C) a large IPSP was produced A preceding conditioning stimulation of L5 and L6 ventral roots virtually abolished this IPSP (D) The effect from the ventral roots was exerted without any recurrent IPSP in the motoneurone The facilitation of Ia IPSPs from the Deiters' nucleus is due to a monosynaptic excitation onto the Ia inhibitory interneurones from the vestibulospinal tract (Grillner *et al* 1966) and the recurrent depression of Ia IPSPs is caused by postsynaptic inhibition of the Ia inhibitory interneurones (Hultborn *et al* 1971 [illegible]) Thus it is concluded that a convergence of vestibulospinal excitation and recurrent inhibition occurs onto interneurones in the Ia inhibitory pathway

Such a convergence was investigated in 28 motoneurones (Table I [illegible]) in which facilitation of Ia IPSPs by volleys in the vestibulospinal tract as well as the recurrent inhibition of Ia IPSPs were present An unequivocal [illegible]

TABLE I Convergence of excitation from the vestibulospinal tract and inhibition from motor axon collaterals in the Ia inhibitory pathway The species of motoneurone investigated are indicated in the upper most horizontal row The motoneurones were divided into two classes conclusive for motoneurones in which an unequivocal convergence in the Ia inhibitory pathway was established and inconclusive for motoneurones in which the efforts to show this convergence failed The figures in the columns for the different species of motoneurone indicate the distribution of tested motoneurones between these two classes The right hand column show the total number of motoneurones investigated *and their distribution among the two classes*

motor nuclei	PBSt	AB	
number of cells tested	23	5	28
convergence			
conclusive	19	4	23
inconclusive	4	1	5

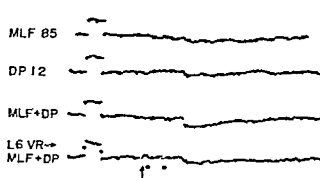


Fig 3 Facilitation from MLF of the transmission in the Ia inhibitory pathway to an ankle extensor G—S motoneurone All traces are *averaged intracellular responses* The points below the lowermost trace indicate time when the two MLF stimuli were delivered The strength of MLF stimulation is given in μA The time of stimulation of L6 VR is marked by the arrow The calibration pulses at the beginning of the averaged records have an amplitude of 0.2 mV and a duration of 4 0 ms

these 28 motoneurones In five motoneurones classified as inconclusive in table I the descending facilitation and/or recurrent depression of the Ia IPSPs were not large enough to prove a convergence onto the interneurones although such a convergence could by no means be excluded

2 *A reticulospinal tract activated in the medial longitudinal fascicle (MLF)* A presumed reticulospinal tract activated in MLF (Grillner and Lund 1968) gives disynaptic IPSPs in knee and ankle extensor motoneurones (Grillner *et al* 1971) On a few occasions weak facilitation of Ia IPSPs to knee and ankle extensor motoneurones was recognized on stimulation of the MFL (Grillner *et al* 1968) During the present investigation we were able to confirm this facilitation of Ia IPSPs on stimulation of the MLF only in 3 G S motoneurones of 41 motoneurones tested (7 G S and 34 Q The most clear example is illustrated in Fig 3 The stimulation of MLF alone did hardly evoke any PSP and the stimulation of the nerve from the antagonist muscle DP only gave rise to a very small Ia IPSP When they were stimulated together (MLF + DP a facilitation was revealed A preceding conditioning volley in L6 ventral root depressed the facilitated IPSP (lowermost trace)

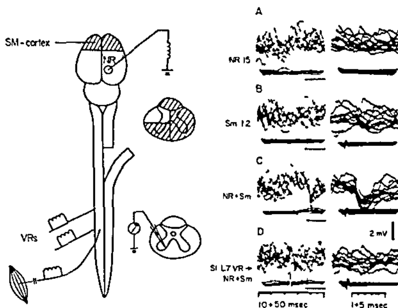


Fig 4 Convergence of excitation from the rubrospinal tract and inhibition from motor axon collaterals in the Ia inhibitory pathway to a hip flexor Sart motoneurone Upper traces in A—D are intracellular records Lower traces are from L6 dorsal root entry zone The right and left pair of records in A—D were taken simultaneously at a slow and fast sweep speed respectively The expanded part of the slow records is indicated by a dashed line The strength of stimulation of the red nucleus is given in μA Voltage calibration for intracellular recordings Time calibrations given below records in D are for the slow and fast records respectively A schematic drawing of the experimental arrangement is shown in the left part of the figure (further description under Methods)

but this decrease was not large enough to allow any conclusion whether a convergence of excitation from MLF and recurrent inhibition occurred onto the same Ia inhibitory interneurones Corresponding findings were made in the remaining two G S motoneurones in which the Ia IPSP could be facilitated

3 *The rubrospinal tract* Volleys in the rubrospinal tract facilitate at an interneuronal level the transmission of Ia IPSPs to motoneurones supplying flexors as well as extensors (Hongo *et al* 1969 b) The segmental latency may occasionally be so brief that a monosynaptic action on the Ia inhibitory interneurones cannot be excluded, but it is much more likely that the minimal linkage is disynaptic Most often a train of stimuli is required to evoke a facilitation of the Ia IPSPs and the segmental latency from the first effective stimulus then usually indicate a di- or trisynaptic excitatory action from the rubrospinal tract onto Ia inhibitory interneurones The experimental procedures were chosen to assure that the effects of stimulation of the red nucleus was mediated by the rubrospinal tract (see Hongo *et al* 1969 a, Baldissera, Lundberg and Udo 1971, and under Methods) A convergence of rubrospinal excitation and recurrent inhibition on interneurones in the Ia inhibitory pathway has now been found as illustrated in Fig 4 and 5 Fig 4 is

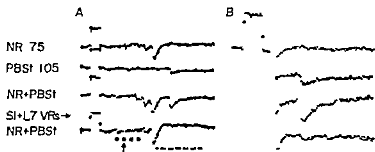


Fig 5 Convergence of excitation from the rubrospinal tract and inhibition from motor axon collaterals in the Ia inhibitory pathway to a knee extensor Q motoneurone All traces consist of averaged intracellular records The strength of stimulation of the red nucleus is given in μA The points below the lowermost trace indicate when the stimuli in the red nucleus were delivered The time of stimulation of L7+S1 VRs is marked by the arrow The dashed line below the slow records in A show the part expanded in B The calibration pulses have an amplitude of 0 5 mV and a duration of 4 0 ms

TABLE II Convergence of excitation from the rubrospinal tract and inhibition from motor axon collaterals in the Ia inhibitory pathway Further description in Table I

motor nuclei	ABSm	Q	G S	Sart	PBSt	DP	
number of cells tested	3	21	6	1	13	11	55
convergence							
conclusive	3	15	6	1	12	9	46
inconclusive	0	6	0	0	1	2	9

)

from a hip flexor Sart motoneurone Records in A and B show hardly any IPSPs to separate stimulation of the red nucleus and Sm nerve while a large IPSP was evoked by conjoined stimulation (C) When preceded by a conditioning volley in S1 and L7 ventral roots the latter IPSP was virtually abolished (D) Fig 5 illustrates a similar convergence for a knee extensor Q motoneurone The stimulation of the red nucleus itself evoked disynaptic IPSPs Despite this effect evoked by stimulation of the red nucleus alone it can easily be seen that the Ia IPSP from the PBSt nerve was facilitated when preceded by stimulation of the red nucleus (NR+PBSt) An antidromic conditioning volley in S1 and L7 ventral roots almost abolished the facilitated IPSP thus proving the convergence of rubrospinal excitation and recurrent inhibition also on Ia inhibitory interneurones destinated to an extensor motoneurone

Our material consists of 52 motoneurones to flexors and extensors at different joints (Table II) in which the rubrospinal facilitation of Ia IPSPs (Hongo *et al* 1969 b) and the recurrent depression of Ia IPSPs (Hultborn *et al* 1971 a) was confirmed In 43 of these 52 motoneurones a clear convergence was established while 9 were classified as inconclusive (for criteria see above)

4 *The corticospinal tract* Facilitation of transmission in the Ia inhibitory pathway to motoneurones from the sensorimotor cortex is mediated by the pyramidal tract in

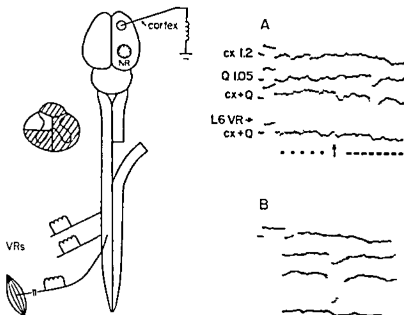


Fig 6 Convergence of excitation from the corticospinal tract and inhibition from motor axon collaterals in the Ia inhibitory pathway to a knee flexor PBSt motoneurone All traces consist of averaged intracellular records The stimulus strength of cortex is given in mA The points below the lowermost trace in A indicate when the stimuli to the sensorimotor cortex were delivered The time of stimulation of L6 VR is marked by the arrow The dashed line below the slow records in A show the part which is expanded in B The calibration pulses have an amplitude of 0 5 mV and a duration of 4 0 ms A schematic drawing of the experimental arrangement is shown in the left part of the figure (further description under Methods and in text)

nembutalized cats (Lundberg and Voorhoeve 1962) It occurs with a long segmental latency that is not compatible with a monosynaptic action on the Ia inhibitory interneurones from the fastest corticospinal axons Under chloralose or very light Nembutal anesthesia it is possible to evoke a facilitation of reflex transmission to motoneurones (including a facilitation of Ia IPSPs) from the sensorimotor cortex also in cats with transected pyramids (Hongo and Jankowska 1967) This facilitation of Ia IPSPs is probably mediated via the rubrospinal tract though reticulospinal paths are also activated by the cortical stimulation (Hongo and Jankowska 1967) It should be noted that the best cortical areas for the pyramidal and extrapyramidal effects are different, the pyramidal effects to the hindlimb segments are most potently elicited from the most medial part of the postsigmoid gyrus just posterior to the cruciate sulcus while the extrapyramidal effects are evoked from the area around the postcruciate dimple which is lateral and slightly posterior to the hindlimb area for pyramidal effects (Hongo and Jankowska 1967 *cf* also Lundberg and Voorhoeve 1962)

The red nucleus was either destroyed by electrolytic lesions or the rubrospinal tract was transected at a medullary level in order to avoid cortico-rubro spinal effects and

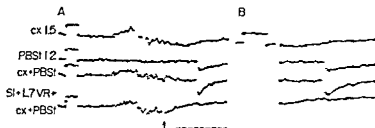


Fig 7 Convergence of excitation from the corticospinal tract and inhibition from motor axon collaterals in the Ia inhibitory pathway to a knee extensor Q motoneurone All traces are averaged intracellular records The stimulus strength of cortex is given in mA The points below the lowermost trace in A indicates when the stimuli to the sensorimotor cortex were delivered The time of stimulation of S1 + L7 VRs is marked by the arrow The dashed line below the slow records in A show the part which is expanded in B The calibration pulses have an amplitude of 0 5 mV and a duration of 4 0 ms

TABLE III Convergence of excitation from the corticosp nal tract and inhibition from motor axon collaterals in the Ia inhibitory pathway Further description in Table I

motor nuclei	ABSm	Q	G S	FDL	PBSt	DP	
number of cells tested	5	7	1	1	22	4	40
convergence							
conclusive	4	6	1	1	19	3	34
nconclusive	1	1	0	0	3	1	6

Nembutal anesthesia was used to depress transmission via the cortico reticulo spinal pathways (see Hongo and Jankowska 1967) Only the dorsal quadrant of the lateral funicle (including the corticospinal tract) was left intact (see Methods and Fig 6) Moreover it was found that in this preparation the lowest thresholds for facilitation of Ia IPSPs were located in the hindlimb area for pyramidal effects Accordingly there is no doubt that the effects investigated were mediated by the corticospinal tract

A convergence of corticospinal excitation and recurrent inhibition on individual Ia inhibitory interneurones is illustrated in Fig 6 which shows records from a knee flexor PBSt motoneurone Stimulation of cortex alone gives almost no PSP and weak stimulation of the nerve to the antagonist Q only evoked a small Ia IPSP When they were stimulated together a large IPSP was evoked due to a spatial summation A conditioning ventral root stimulation almost abolished the IPSP thus proving the convergence of (polysynaptic) pyramidal excitation and recurrent inhibition on individual Ia inhibitory interneurones Fig 7 illustrates a similar convergence in a knee extensor (Q) motoneurone Thus transmission in the Ia inhibitory pathway to flexor and extensor motoneurones are subject to a similar control The convergence of corticospinal excitation and recurrent inhibition was analyzed in 40 motoneurones (Table III) in which the corticospinal facilitation of Ia IPSPs (Lundberg and

Voorhoeve 1962) and the recurrent depression of Ia IPSPs (Hultborn *et al* 1971 a) was confirmed In 34 of these 40 motoneurones a clear convergence was found while 6 were classified as 'inconclusive' (for criteria see above)

Discussion

There are now many observations showing that interneurones of reflex pathways to α motoneurones receive extensive convergence from other neuronal pathways than the primary afferents by which they are designated (*cf e g* Lundberg 1969) The present investigation deals with a problem raised by the very finding of such an extensive convergence at an interneuronal level is a given reflex pathway functionally homogeneous—in other words is there a similar convergence on all its interneurones —or does it have functional subdivisions characterized by differences in interneuronal convergence? Our present information regarding convergence on interneurones of reflex pathways rests largely on studies of how the synaptic potentials evoked in motoneurones from primary afferents are influenced from other neuronal systems An excitatory convergence is indicated by facilitation and an inhibitory by depression of the test PSP This technique has now been modified in order to allow deductions regarding convergence on interneurones from more than two systems A test PSP is evoked by spatial facilitation between two excitatory convergent systems and the depression of this test PSP from an inhibitory system allows the conclusion that the three systems involved converge onto the same interneurones

In the present investigation of the Ia inhibitory pathway we have thus established that excitation from either corticospinal rubrospinal or vestibulospinal pathways on one hand and recurrent inhibition from motor axon collaterals on the other do converge onto the same Ia inhibitory interneurones

In case of the reticulospinal system activated by stimulation of MLF we were not able to demonstrate a similar convergence In fact the facilitation of Ia inhibition following stimulation of MLF has proved extremely rare Even in the few cases seen it has been very weak and the MLF system can therefore hardly be considered to exert any regular excitatory action on Ia inhibitory interneurones Our failure to show the convergence of excitation from MLF and inhibition via the recurrent pathway on common Ia inhibitory interneurones (in cases when facilitation of Ia IPSP from MLF was present) may only reflect experimental difficulties caused by weak facilitatory action from MLF and a weak recurrent depression of the trans-

ding on that the most effec
since they also evoke large
a convergence of excitation
Ia inhibitory interneurones
can certainly not be excluded on the basis of our material The unequivocal findings of such a convergence for the vestibulo- rubro- and corticospinal systems make it likely to exist also for the MLF system when activity in th s pathway at all excite the Ia inhibitory interneurones

Although the present investigation has proved the existence of Ia inhibitory interneurones receiving both supraspinal excitation and recurrent inhibition the method employed does not allow a quantitative evaluation of the occurrence of such interneurones We can thus not exclude the possible existence of other groups of Ia inhibitory interneurones receiving either descending excitation or recurrent inhibition but not both

For most polysynaptic pathways a study of their synaptic potentials in motoneurones is the only way to obtain information regarding the interneuronal organization because the gain of the more direct approach of recording the interneurones themselves often is hampered by the difficulties of identifying them as belonging to a defined pathway (see Lundberg 1969) In case of the reciprocal Ia inhibitory pathway the present knowledge obtained by the indirect approach (*cf* Eccles 1964 Lundberg 1970 Hultborn *et al* 1971 a) have permitted Hultborn Jankowska and Lindstrom (1971 b) to postulate that the Ia coupled interneurones which are located in the ventral horn just dorsomedial to the motor nuclei are mediating the reciprocal Ia inhibition In fact interneurones identified with those criteria were recently shown to produce monosynaptic IPSPs in motoneurones (Jankowska and Roberts 1971) thus finally proving that they are the interneurones interposed in the disynaptic Ia inhibitory pathway to motoneurones

The descending effects on these Ia coupled interneurones in the ventral horn have only been investigated with stimulation of dissected spinal fascicles (Hultborn *et al* 1971 b) It is however of interest that monosynaptic EPSPs were evoked from the ipsilateral ventral quadrant of the spinal cord in interneurones excited by volleys in Ia afferents from extensors (Hultborn Jankowska and Lindstrom 1968 Hultborn *et al* 1971 b *cf* also Skinner Willis and Hancock 1970) These monosynaptic EPSPs may well originate from the vestibulospinal tract fibres (*e g* Nyberg Hansen and Mascitti 1964) which selectively facilitates the transmission in the Ia inhibitory pathway from extensors to flexors (Grillner *et al* 1966) Excitation from the dorsal quadrant of the spinal cord or from the contralateral spinal half was observed in a few interneurones but with longer latencies suggesting at least a disynaptic linkage Further studies on the Ia interneurones in the ventral horn with stimulation of defined descending systems will show if these Ia interneurones are subject to all descending actions postulated for the interneurones mediating the reciprocal Ia inhibition Such a study will also answer the question whether individual interneurones are subject to a descending excitatory control from more than one supraspinal structure a question not readily solved by any other approach

Since the main aim of this investigation has been to elucidate whether a convergence of descending excitatory and recurrent inhibitory actions occur on the same Ia inhibitory interneurones it may be worth to consider possible interpretations of such a convergence The finding that impulses in motor axon collaterals inhibit in parallel motoneurones and inhibitory interneurones supplied by the same Ia afferents led Hultborn Jankowska and Lindstrom (1971 c) to postulate a functional unity between the recurrent control of motoneurones and Ia inhibitory interneurones Since the recurrent inhibition thus was found to be related to the Ia connexions it was suggested that the recurrent inhibition may have its special significance in controlled movements Many descending pathways in fact all so far investigated have been shown to evoke parallel effects on α and motoneurones (*cf* Granit 1968 1970 Grillner 1969) thus conforming with the idea that movements depend on an α, linkage as postulated by Granit (1955) According to this hypothesis a neuronal

system will exert synaptic depolarization of α motoneurones via two routes—the direct α route and the indirect γ route the latter giving depolarization of α motoneurones across the γ loop The idea with an α γ-linkage implies that the discharge of many α motoneurones will depend on the spatial facilitation from these two pathways Hongo, Jankowska and Lundberg (1969 b) forwarded the hypothesis that essentially the same mechanism operates in the control of reciprocal inhibition and thus introduced the term 'α γ linked reciprocal inhibition It was pointed out (Hongo *et al* 1969 b Lundberg 1970) that the convergence of Ia and descending impulses will allow the servo properties of the stretch reflex to govern also the reciprocal inhibition of antagonists Although the α γ linkage in the control of reciprocal inhibition must be accomplished by convergence at the interneuronal level this integrative control of reciprocal inhibition was regarded functionally equivalent to the α γ-linkage at the motoneuronal level in excitation of motoneurones

If the concept of 'α γ linked reciprocal inhibition is correct our present findings should be interpreted in terms of a recurrent control from motor axon collaterals of 'α γ linked reciprocal inhibition', independently of whether this reciprocal inhibition is produced by activity in the vestibulospinal rubrospinal or corticospinal pathways

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Uptake of Atropine and Methylatropine by Immature Choroid Plexus in vitro*

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Abstract

WINBLADH, B *Uptake of atropine and methylatropine by immature choroid plexus in vitro* Acta physiol scand 1972 *84* 109—114

Choroid plexa from newborn rabbits accumulate atropine and methylatropine *in vitro* by a mechanism which fulfils the requirements for active transport. Tissue medium ratios at a fixed concentration increase from 3 days before term until 2 weeks of age, when adult levels are reached Uptake maximal velocity per unit tissue wet weight (V_{max}) increases with age, while apparent K_m remains constant The conclusion is drawn that the same carrier mechanism is involved in the newborn and adult tissues The change in V_{max} is discussed in relation to developmental changes in tissue composition and morphology Further possible *in vivo* significance of the *in vitro* findings is discussed

It has been shown that certain substances including some quaternary amines (Schanker *et al* 1962) are cleared out of the cerebrospinal fluid (CSF) partly by a carrier mediated mechanism This is possibly also the case with atropine (Albanus *et al* 1969) Choroid plexa *in vitro* accumulate these substances by a mechanism which fulfils the requirements for active transport (Tochino and Schanker 1965, Winbladh 1971) It thus seems possible that the carrier mediated CSF clearance mechanism at least partly is localized to the choroid plexus Since we at present are studying changes in atropine and methylatropine metabolism and sensitivity with age, we considered it of interest to investigate if any changes in choroid plexus accumulation of these two drugs occurred during maturation

Methods

Small Chinchilla rabbits were used at five different ages i e 28 days old fetuses 3 days before term), newborn (2—12 h), 42—54 h four days and 12—14 days old The fetuses were delivered by caesarian section after the mother had been killed with air i v After delivery the fetuses showed good vitality The animals were sacrificed by decapitation the choroid plexa from the lateral ventricles were rapidly dissected out, rinsed in ice-cold Krebs-Henseleit buffer

* A preliminary report was presented at the 24th Scandinavian Pharmacological Meeting Oslo, 1970 (B Windbladh 1970)

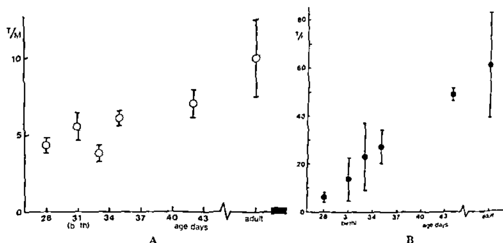


Fig 1 Variation of choroid plexus uptake with age T/M = tissue medium ratio, concentration of drug in incubation medium 10^{-4} M A Atropine B Methylatropine

and transferred to chilled Warburg flasks with 2 ml of the buffer Incubation with tritium labelled atropine[1] or methylatropine[2] with necessary amounts of unlabelled drug was performed at 37° C gassing with 93 5 % O_2 and 6 5 % CO_2 and gentle shaking in a Warburg apparatus After incubation the tissue was rinsed in ice cold Krebs Henseleit buffer and excess solution removed with filter paper The tissue was then immediately weighed on a microbalance special care being taken to avoid drying of the tissue After drying to constant weight tissue drug content was assayed by liquid scintillation counting after combustion as described elsewhere (Winbladh 1971) Incubation medium drug content was also determined by liquid scintillation counting Tissue medium ratio (T/M) was determined as the quotient between tissue dpm per gram wet weight and incubation medium dpm per ml

Protein determinations were performed with the method of Lowry *et al* 1951 Choroid plexus potassium content was analyzed by homogenizing the tissue in 0 5 mg of 1 M HNO_3 after incubation After centrifugation (15 000 g × min) the pellet was washed twice with 1 M HNO_3 and the pooled supernatants analyzed for their K content by flame photometry (EEL England)

Calculations of maximal velocity (V_{max}) and apparent K_m was performed as described elsewhere (Eriksson and Winbladh 1971) Decrease in incubation medium drug content caused by tissue uptake did not exceed 15 %

Results

At the 6 ages investigated both atropine and methylatropine is accumulated in choroid plexus against a concentration gradient (Fig 1) T/M increases with incubation time with tissue from newborn animals and the increase is reasonably linear during the first 40 min (Fig 2 The kinetic constants have been obtained at an incubation time of 30 min for atropine and 40 min for methylatropine Uptake is

[1] T Atropine 434 mCi/mmol generally labelled purchased from The Radiochemical Centre Amersham, Great Britain

[2] T Methylatropine was made by quaternization of T atropine with methylatropine in acetonitrile during 30 min followed by evaporation of excess methyliodide and solvent The obtained product moved in one peak indistinguishable from methylatropine in paper electrophoresis (50 V/cm, Whatman no 1 0 1 M borate sodium hydroxide buffer pH 10) and thin layer chromatography (methanol acetate acid water 3 1 1, Merck DC Fertigplatten Silica F_{254})

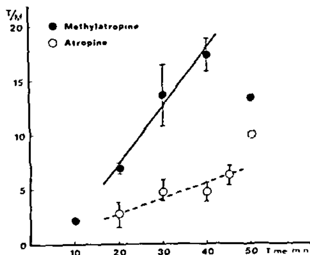


Fig 2 Variation of choroid plexus uptake with time T/M = tissue medium ratio, mean with limits Concentration of drug in incubation medium 10^{-4} M

TABLE I Inhibition of Choroid Plexus Uptake of Atropine and Methylatropine at 0° C

	Inhibition %
Atropine	87, 85
Methylatropine	98 98

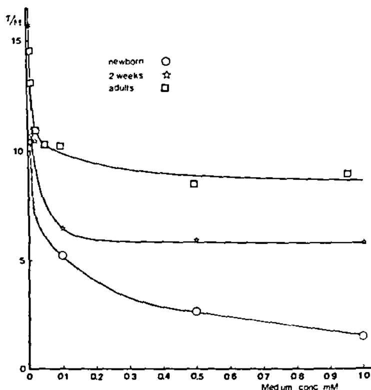
TABLE II Changes in Uptake Characteristics of Choroid Plexus with Age

Age	Atropine			Methylatropine		
	Number of expts	$V_{max} \pm SE$ mmol/min × kg	$K_m \pm SE$ mM	Number of expts	$V_{max} \pm SE$ mmol/min × kg	$K_m \pm SE$ mM
Newborn	11	0.15 ± 0.01	0.95 ± 0.15	18	0.05 ± 0.01	0.06 ± 0.02
2 weeks	14	0.28 ± 0.05	0.93 ± 0.29	14	0.27 ± 0.03	0.10 ± 0.03
Adult*	40	0.35 ± 0.02	0.93 ± 0.12	30	0.26 ± 0.02	0.08 ± 0.02

* From Eriksson and Winbladh 1971

dependent on drug concentration in the incubation medium and shows saturation characteristics at the two ages studied in this respect *i e* newborn and 2 weeks (Fig 3) Further with tissue from newborn animals accumulation is greatly depressed by incubation at 0° C (Table I)

When choroid plexa from animals of different ages are incubated at a fixed concentration of 10^{-4} M, there is an increase in T/M with age as seen from Fig 1 The changes with age in calculated V_{max} and apparent K_m are shown in Table II Values for adult animals are taken from an earlier report (Winbladh and Eriksson 1971) The data suggest that the change in T/M at a certain concentration is due to a change in V_{max} while K_m remains relatively constant There is a fairly large



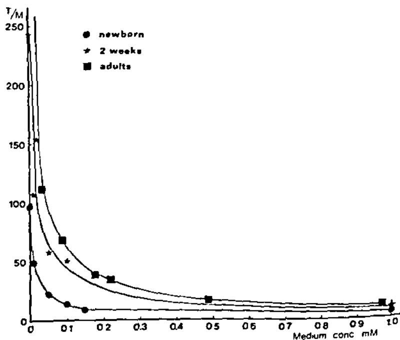


Fig 3 Influence of incubation medium concentration on choroid plexus uptake of atropine (A) and methylatropine (B) T/M – tissue medium ratio mean values

Table III Variation of Choroid Plexus with Age*

Age	Wet weight mg±SD (n)	Water content %±SD (n)	Wet weight / brain wet weight ×10³	Protein content %±SD (n)	Potassium content mg/g wet weight ±SD (n)
3 days before term	1 3±0 3 (9)	74±5 (9)	1 51	—	—
newborn	1 4±0 2 (9)	83±5 (12)	1 21	10 9±2 2 (3)	6 4±1 1 (6)
2 days	1 8±0 2 (5)	75±3 (6)	1 09	—	—
4 days	1 9±0 6 (6)	77±2 (5)	1 07	—	—
2 weeks	3 6±0 9 (8)	76±9 (15)	1 07	—	—
adult	8 3±2 1 (79)	78±3 (15)	0 94	11 0±1 0 (4)	7 5±0 7 (3)

* Figures refere to one telencephalic choroid plexus
n = number of experimenty

scattering of data giving considerable standard errors in K_m This scattering has been the experience also with adult animals (Welch 1962, Tochino and Schanker 1965, Winbladh and Eriksson 1971)

Relative water content of choroid plexus determined by dry-wet weight ratio and protein content does not change significantly with age as shown in Table III, while mean potassium concentration is slightly higher in the adult animals Routine histological examination[3] of the tissue before and after incubation revealed no significant morphological differences

Discussion

In the newborn rabbit choroid plexus uptake of atropine and methylatropine fulfils the conventional criteria for active transport *i e* accumulation against a concentration gradient by an energy dependent, saturable mechanism It thus seems highly probable that the accumulation also for the other age groups depends on active transport, since that has been shown to be the case in adult rabbits (Eriksson and Winbladh 1971)

In vitro there is an increase in V_{max} and also a change in apparent K_m for the two drugs with age However, the differences in apparent K_m are small and probably not significant This suggests that the same carrier mechanism is involved in the newborn tissue as in the adult A decrease in extracellular volume (ECV) of choroid plexus with increasing age would cause an increase in V_{max} Because of the ability of choroid plexus to accumulate proteins (Smith *et al* 1964) and its pinocytotic activity (Tennyson and Pappas 1961) it seems difficult to obtain a reliable estimation of ECV with the usual extracellular tags It has been shown that intracellular potassium concentration in dog cat, guinea pig and rat striated muscle and cat brain and liver does not change significantly with age (Kerpel Fronius *et al* 1964 Yannet and Darrow 1938) Assuming this holds for rabbit choroid plexus the potassium contents given in Table III should roughly reflect ECV The difference between new-

[3] Formaldehyde fixation paraffin imbedding and haematoxylin-eosin or van Gieson staining

born and adult animals is hardly significant and anyway cannot explain the dif ference in V_{max}.

The *in vivo* meaning of these age differences in uptake velocity is difficult to assess It seems reasonable to assume that the transport mechanism operates in ependymal-endothelial direction (for discussion see Eriksson and Winbladh 1971) Anatomical and physiological parameters such as choroid plexus weight in relation to ventricular volume, choroid plexus localization and blood flow in relation to CSF production and flow would all modify the *in vivo* effect of a choroid plexus transport No data are available about changes in these parameters, but choroid plexus wet weight seems to decrease with age in relation to the wet weight of the whole brain (Table III) *In vitro* data need thus not always reflect *in vivo* conditions Robinson *et al* (1968) reported that T/M for cat choroid plexus uptake of inorganic sulphate was low prenatally, showed a peak value at birth and then declined However, CSF sulphate clearance has been reported to be faster in the adult cat than in the kitten (Cutler *et al* 1968)

According to the electronmicroscopic investigations by Tennyson and Pappa (1961) there is a rapid development of intracellular structures associated with active transport in the rabbit choroid plexus from late foetal stage to 14 days postnatally At this age the tissue has an almost mature appearance which is in good agreement with our findings

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Effect of 6-Hydroxydopamine on the Ganglion Cells and the Small Intensely Fluorescent Cells in the Superior Cervical Ganglion of the Rat

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Abstract

ERÄNKO, L and O ERÄNKO *Effect of 6-hydroxydopamine on the ganglion cells and the small intensely fluorescent cells in the superior cervical ganglion of the rat.* Acta physiol scand 1972 *84* 115—124

Newborn rats were injected daily for 8 days with 6-hydroxydopamine (6 OH DA) 50 mg/kg b w Disappearance of over 90 % of the ganglion cells from the superior cervical ganglion and the coeliac ganglion was observed 3 weeks after discontinuation of the injections The surviving ganglion cells were larger than normal and exhibited less intense than normal catecholamine fluorescence but showed normal or slightly [illegible]
specific chol[illegible]
the Golgi ap[illegible]
increase in [illegible]
was due to [illegible]
intensely fluorescent cells counted from complete series of sections through the ganglia was the same in controls as that in animals injected with 6 OH DA It is concluded that the small intensely fluorescent cells are resistant to chemical sympathectomy and differ in this respect from ordinary ganglion cells

Administration of 6-hydroxydopamine (6-OH DA) causes, in an adult animal, a reversible destruction of peripheral adrenergic nerve terminals without affecting the nerve cell body (Tranzer and Thoenen 1967 1968) In newborn animals 6 OH-DA has been reported to cause a life long chemical sympathectomy, i e, irreversible degeneration of the peripheral adrenergic nerve terminals and permanent destruction of sympathetic ganglia (Angeletti and Levi-Montalcini 1970)

Different types of sympathetic ganglion cells have been described on the basis of their cholinesterase activity and catecholamine fluorescence (see Härkönen 1964 Eränko 1967 a), moreover small intensely fluorescent (SIF) cells are present in the sympathetic ganglia whose properties differ from those of ordinary ganglion ce[illegible] (Eränko and Härkönen 1963, 1965, see also reviews by Jacobowitz 1970 and Er[illegible] and Eränko 1971 a) To our knowledge, neither cholinesterases and ca[illegible]

nor SIF cells have been examined in sympathetic ganglia of animals treated with 6-OH-DA Such a study is reported in this paper, together with some observations on the changes in the fine structure of the ganglion cells

Material and Methods

Experimental

Newborn rats were injected within 12 h after birth intraperitoneally with 6 OH DA (a gift kindly supplied by Messrs F Hoffmann La Roche Basel) 50 mg/kg b w a similar injection was thereafter given daily in t[illegible] Angele[illegible] and Levi Montalcini (1970) for [illegible] of the rat A fresh solution [illegible] of 6 OH DA in 1 ml of 0 [illegible] in each ml

Of each litter, one half of the rats were injected, while the other half served as untreated controls The injected rats altogether 16 animals were killed together with the same number of untreated litter mate controls at the age of about 1 month, when 3 weeks had elapsed after the last injection of 6 OH DA The superior cervical and coeliac ganglia were removed immediately after killing

Demonstration of catecholamines

The fresh ganglia were placed on an aluminium foil and frozen on it by immersion in propan[illegible] cooled to $-190°$ C with liquid nitrogen Freeze-drying was carried out for a week at $-45°$ [illegible]

[illegible] continued for 1 h For details of the technique the reader is referred to Eränkö (1967 b)

The formaldehyde treated ganglia were embedded in a mixture of Epon and Araldit[illegible] (Eränkö and Eränkö 1971 a) and cut into complete series of 5 μm thick sections with a glas[illegible] knife using the LKB Pyramitome The sections were transferred dry on a slide and mounted i[illegible] Entellan (E Merck Darmstadt) For fluorescence microscopy of catecholamines an Ortholu[illegible] microscope (E Leitz Wetzlar) was with an Osram HBO 200 high pressure mercury lamp tw[illegible] 3 mm thick BG 38 filters one 3 mm thick BG 3 and one TAL 405 interference filter (Schott et Gen Mainz) and with the Ploem (1971) epilluminator with dichroic mirrors Above the objective the ultraviolet absorbing filter K 470 (Leitz) was used

For counting of the SIF cells all sections cut from the superior cervical ganglia were examined The section thickness error was corrected using the Floderus formula (see Eränkö 1955)

Demonstration of cholinesterases

The ganglia were fixed by immersion as whole in an ice cold 3 5 % solution of formaldehyde in 0 1 M phosphate buffer pH 7 4 The formaldehyde was freshly made by dissolving para formaldehyde with sufficient dropwise addition of sodium hydroxide solution to bring the paraformaldehyde into solution After fixation overnight the ganglia were rinsed, again over night in 0 1 M phosphate buffer pH 7 4 with 0 3 M of sucrose Thereafter frozen sections were cut at 10 μm The sections were incubated immersed in the substrate mixture containing acetylthiocholine and made according to Gomori s (1952) transcription of Koelle s (1951) original recipe

Before incubation the sections were pre incubated for 30 min in 10^{-5} M solution of tetra isopropylpyrophosphoramide iso-OMPA I Light Colnbrook) a specific inhibitor of non specific cholinesterase (butyrylcholinesterase) when acetylcholinesterase was demonstrated The same inhibitor was included in the same concentration in the incubation solution containing the substrate

For the demonstration of non specific cholinesterase the pre incubation and incubation were carried out in the same way but using instead of iso-OMPA 1 5 bis (4 allyl dimethylammoniumphenyl) pentan 3 one diiodide (284C51 Burroughs and Wellcome London) a specific inhibitor of acetylcholinesterase in a concentration of 10^{-5} M and butyrylthiocholine as a substrate The efficiency of the inhibitors towards the cholinesterase activities in the sympathetic ganglia was tested and the above concentrations proved suitable (see also Eränkö et al 1964)

Electron microscopy

Small pieces of the g
The pieces were ther
in granular ice The
phosphate buffered K
ganate fixative first
formaldehyde in 0 1 M

Fixation was carried out for 1 h in permanganate and the tissues were then rinsed overnight in the Krebs-Ringer solution at 0° C dehydrated and embedded in a mixture of Epon and Araldite (Eranko and Eranko 1971 a) After fixation in the glutaraldehyde-formaldehyde mixture the tissues were rinsed overnight in the Krebs Ringer solution at 0° C re fixed in an 1 % solution of osmium tetroxide in 0 1 M phosphate buffer, pH 7 4 at 0° C for 1 hour, rinsed in 0 1 M phosphate buffer for 30 min as well as dehydrated and mounted in the Epon Araldite mixture as described above Thin sections cut with the LKB Ultrotome were examined with the Philips EM 300 electron microscope at 40 or 60 kV

Results

General observations

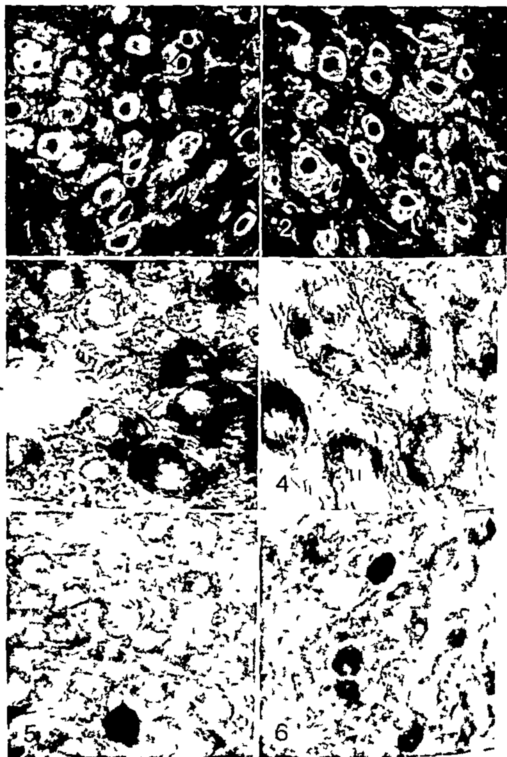
The general appearance of the rats which had been injected with 6-OH DA was essentially similar as that of their untreated litter mate controls However, they weighed about 20 % less than the controls No differences in the behaviour or spontaneous activity were observed

The size of the superior cervical ganglion and that of the coeliac ganglion were drastically reduced in the animals injected with 6-OH DA The diameter of the superior cervical ganglion of injected rats was reduced to less than one third of original, which indicates a volume diminution to less than about one tenth of the control value In some of the 6-OH DA injected animals, small rounded nodules were observed in the slender ganglia of the injected rats

Catecholamine fluorescence

Fig 1 shows the distribution of the formaldehyde induced fluorescence due to nor adrenaline in the superior cervical ganglion of an 1 month-old control rat The ganglion cell bodies exhibited a diffuse cytoplasmic fluorescence, whose intensity varied from one ganglion cell to another In addition to the diffuse cytoplasmic fluorescence, fluorescent granules were seen in the peripheral parts of the perikaryon In the normal ganglia the cells were fairly close to each others although there was a distinct intercellular space between the cell bodies filled with both non fluorescent and fluorescent cell processes A small group of SIF cells is also visible in the left upper corner of Fig 1

Fig 2 is of an identically made preparation from the nodular part of the superior cervical ganglion of an 1 month old rat injected daily for 8 days with 50 mg of hydroxydopamine per kg b w The general view is not much different from that of the normal ganglion illustrated in Fig 1 although most of the cells exhibit only a relatively weak fluorescence the variation between the individual cell bodies being thus smaller than in normal controls The relative amount of intercellular tissue was distinctly increased and several abnormally large cells were seen One such cell is above the number 2 in Fig 2 Since over 90 % of the original ganglion had com



(For legends see opp   1 s

TABLE I Effect of 6-hydroxydopamine on the number of small intensely fluorescent (SIF) cells in the superior cervical ganglion of the rat

Group	Number of rats	SIF cells per ganglion	
		Mean	S D
Control	5	466	76
6—OH—DA	5	472	68

pletely disappeared after treatment of 6-OH DA it is understandable that large areas of the sympathetic trunk were also seen in the injected animals in which no ganglion cells were seen at all

The relative number of the SIF cells in the ganglion appeared greatly increased after treatment with 6-OH DA However, the increase was found to be due to concentration of the SIF cells into a smaller volume, when most ordinary ganglion cells which normally separate the clusters of small cells, had become destroyed by the 6-OH DA treatment When the total number of small intensely fluorescent cells was calculated from a complete series of sections about 470 cells were found both in the controls and in the injected animals (Table I)

Cholinesterases

Acetylcholinesterase (AChE) The distribution of AChE activity in a normal ganglion is illustrated in Fig 3 The variation in the AChE activity between individual cell bodies is evident, and the fine cholinesterase positive nerve fibres are clearly visible between the cell bodies

A ganglion from a rat injected with 6-OH DA is shown in Fig 4 Except for a slight increase in the relative amount of intercellular tissue, comparison with Fig 3 also suggests an increase in the average size of the nerve cell bodies and some loss of AChE activity of the cell bodies The rich AChE positive nerve net between the cell bodies is clearly visible

Fig 1 Formaldehyde-induced fluorescence in the superior cervical ganglion of a control rat Individual ganglion cell bodies are close to each others and show a fluorescence of variable intensity 3 SIF cells are visible in the left upper corner × 250

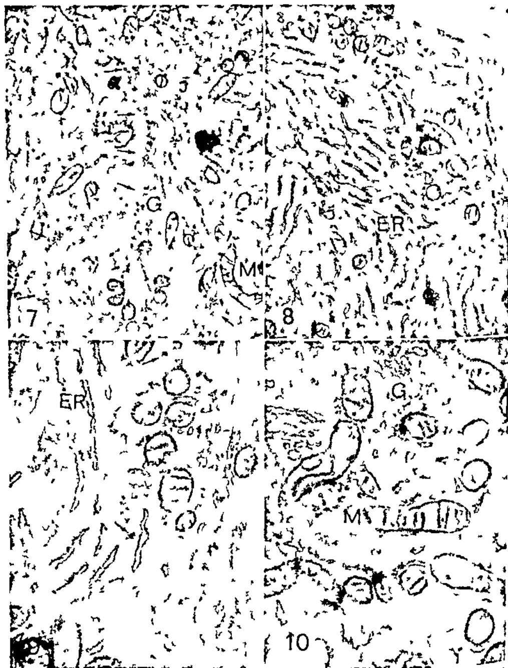
Fig 2 Similar photograph of a rat injected after birth with 6 OH DA Observe large distances between the ganglion cell bodies A hypertrophic cell visible on the lower left part of the figure × 250

Fig 3 AChE *in the superior cervical ganglion of* a control rat Note the variation in th activity between individual ganglion cells × 400

Fig 4 Similar field as that in Fig 3 of a rat treated with 6-OH DA The cells appe l ll and they show a weakened acetylcholinesterase activity Beaded nerve fibres are cl clearly visible in the wide intercellular spaces and on the nerve cell bodies × 100

Fig 5 Non specific cholinesterase activity in the superior cervical ganglion of a ... Most cell bodies show a weak reaction but there is one intensely reactive cell ... centre × 250

Fig 6 Non specific cholinesterase in the same ganglion of a rat treated with ...OH DA The view is essentially similar as that in Fig 5 save for the coincidental prese... 5 intensely reactive cells × 250



Figs 7—10 are all electron micrographs of a typical ganglion cell in the superior cervical ganglion of a rat treated with 6 OH DA. Permanganate fixation no counterstain voltage 40 kV

Fig 7 Part of perinuclear cytoplasm with a well developed Golgi apparatus (G) indicated by arrows M mitochondrion × 20 000

Fig 8 Another field of the periphery of the same cell showing the extensive endoplasmic reticulum (ER) whose numerous ribosomes are not demonstrable with this fixation Arrows indicate two granular vesicles × 20 000

Non specific cholinesterase The distribution of non specific cholinesterase was essentially similar in the ganglia of 6-OH DA treated rats (Fig 6) as that in the ganglia of untreated control rats (Fig 5) although the increase in the size of the cell bodies and in the relative amount of intercellular tissue was again apparent Since it has been shown that cells exhibiting an intense activity of non specific cholinesterase correspond to cells exhibiting a weak catecholamine fluorescence (Harkonen 1964) special attention was paid to the number of such cells possibly cholinergic ones in the ganglia of 6-OH DA treated rats However no significant differences could be observed as compared with normal controls

Electron microscopy

In the normal control rats aged one month the nerve cells of the superior cervical ganglion showed all signs of intense synthetic activity The nuclear membrane was often folded and fitted with several nuclear pores The Golgi apparatus and the granular endoplasmic reticulum were well developed In the peripheral areas of the cytoplasm there were clusters of small granular vesicles Between the cell bodies axons were visible embedded in the Schwann cell cytoplasm and containing alternatively empty vesicles or small granular vesicles

Because of the great variation in the size and the fine structural appearance between individual ganglion cells of normal rats it was difficult to find features in the ganglia of the rats treated with 6 OH DA which had been specifically due to drug administration It was quite clear that whatever cells had survived they also showed well developed and perfectly normal cell organelles Moreover the impression was obtained that in the ganglia of the 6-OH DA treated animals there were signs of an increased cellular activity The endoplasmic reticulum seemed to be more extensive in many cells (Fig 8) and the Golgi apparatus appeared more prominent (Fig 7) However as compared with the control ganglion less small granular vesicles were found in the periphery of the cytoplasm (Fig 8 and 9) All mitochondria (Fig 10) showed normal cristae but they were perhaps longer than those in the control cells Mitochondrial swelling was never encountered in the ganglia of the injected animals and the cristae mitochondriales were well developed

Discussion

The results of the present study show that administration of 6-OH DA dopamine in newborn rats daily for 8 days causes a disappearance of almost all sympathetic ganglion cells from the superior cervical ganglion and the coeliac ganglion This is in agreement with the observation by Angeletti and Levi Montalcini (1970) whose study was carried out mainly with mice although it was briefly mentioned that similar results were obtained with newborn rats.

Fig 9 A detail of the same cell shown in Fig 8 The peripheral rim of the cell contains mitochondria and two granular vesicles (arrows) × 40 000

Fig 10 A view from the central part of the same cell showing typical large mitochondria (M) with intact cristae Part of a Golgi apparatus (G) is also visible × 40 000

However our results differ from those obtained by Angeletti and Levi Montalcini (1970) in so far that not all cells had become destroyed nodules of obviously living ganglion cells being observed in all rats 3 weeks after discontinuation of the 6-OH DA treatment In these animals no such degenerative lesions were seen as were described by Angeletti and Levi Montalcini in the neurons of the injected mice such as disappearance of cell membranes alteration in the mitochondria and lacunar spaces scattered throughout the cytoplasmic area changes which they observed soon after treatment with 6-OH DA Since our material dealt with rats 3 weeks after cessation of the treatment it is indeed likely that changes which had eventually occurred during the treatment of 6 OH DA even in the surviving nerve cells had already disappeared during the recovery period

The ganglion cells which survived the treatment with 6-OH DA differed somewhat from those of untreated controls As judged from the formaldehyde induced fluorescence the catecholamine content of the perikaryon was smaller than that in the controls The size of the cells was increased and there was a tendency towards hypertrophy of the endoplasmic reticulum and the Golgi apparatus as well as an increase in the number of mitochondria These are signs which can also be observed after division of the axon It is therefore of special interest that the changes described by Angeletti and Levi Montalcini (1970) at the early stages of 6-OH DA treatment such as mitochondrial swelling have been observed in the early state of axon reaction (Harkonen 1964) This is noteworthy because 6-OH DA has a more potent action on the axon terminals than the cell body which in adult animal remains unaffected by the treatment The discussed changes in the cell body of animals which have been injected with 6-OH DA since birth suggest that the principal site of its action may be the axon which then causes an axon reaction in the cell body which in some cases is permanent and leads to cell destruction but which is in the minority of cells reversible and compatible with survival and re innervation of peripheral organs such as the iris (Eranko and Eranko 1971 b) However it seems likely that 6-OH DA also directly affects the ganglion cell body especially in the newborn rats whose ganglion cells are yet at a poorly differentiated stage The mechanism proposed by Furness *et al* (1970) for the peripheral axon membrane *i e* uptake of 6-OH DA until a critical concentration is reached and the axon membrane is damaged can be expected to apply also to the nerve cell membrane which is in newborn animals not only less differentiated but also less protected by Schwann cells than in adult ganglia

It is of interest to note that a small nerve cell population ranging in size from 2 to 10 % of the controls persists in sympathetic ganglia of newborn animals immunosympathectomized by injections of antiserum against the nerve growth factor (Levi Montalcini and Angeletti 1966) and these authors propose that these para and prevertebral sympathetic neurons owe their resistance to the antiserum to the fact that they have already attained a considerable degree of differentiation when the treatment was first initiated The size of the cell population which we observed to survive the treatment with 6-OH DA was essentially similar and the same ex

planation can be given for its persistence Knowing that permanent effects of 6-OH DA on sympathetic ganglion cells are also dependent on sufficiently early administration (see the introduction)

It is of special interest that the number of SIF cells in the sympathetic ganglia remains completely unaffected, showing neither increase nor decrease after treatment with 6-OH DA This shows, firstly, that 6-OH DA does not affect the SIF cells in the same way as it does most of the sympathetic ganglion cells, may be because the SIF cells are more differentiated Secondly, it indicates that the destruction of the sympathetic nervous system to the degree of over 90 % does not lead to compensatory hyperplasia of these cells whose number dramatically increases after similar treatment with guanethidine, which also causes a chemical sympathectomy closely resembling that obtained with 6-OH-DA (Eränkö and Eränkö 1971 c) Angeletti and Levi Montalcini (1970) reported hypertrophy of the adrenal medulla two weeks after discontinuation of treatment with 6-OH DA, whose chromaffin cells closely resemble the SIF cells of the sympathetic ganglia (Eränkö and Eränkö 1971 a)

The present study was undertaken after a stimulating discussion with Dr Marthe Vogt for which the authors are very grateful

Skilful technical assistance by Mrs Paula Hasenson and Mrs Tuula Stjernvall is gratefully acknowledged

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Addendum

After the present manuscript was completed the report by Angeletti (1971) was brought to our attention In agreement with our results, he observed 2 weeks and 3, 6 or 10 months *after 6-OH DA administration small nodules with some nerve cells* in the pre- and paravertebral sympathetic ganglia of mice and rats instead of the complete destruction reported in the earlier paper by Angeletti and Levi Montalcini (1970)

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Effect of Hydrocortisone on Histochemically Demonstrable Catecholamines in the Sympathetic Ganglia and Extra-adrenal Chromaffin Tissue of the Rat

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Abstract

ERÄNKÖ, L and O ERÄNKÖ. *Effect of hydrocortisone on histochemically demonstrable catecholamines in the sympathetic ganglia and extra-adrenal chromaffin tissue of the rat* Acta physiol scand 1972 *84* 125—133

[illegible] to newborn rats daily for 5 days, [illegible] were given the same dose daily for [illegible] ere demonstrated by formaldehyde [illegible] d with hydrocortisone there was a [illegible] cells were normally present before hydrocortisone treatment In the adult rats, hydrocortisone did not cause any dramatic changes in the sympathetic ganglia. The SIF cells were present in clusters in the same way as in the control ganglia but there was a tendency towards an increase in the number of SIF cells in the clusters It is concluded that hydrocortisone causes in young rats a greatly increased formation of the SIF cells from poorly differentiated, weakly fluorescent stem cells, while proliferation of already existent SIF cells is less pronounced

Administration of cortisone or hydrocortisone to newborn rats was observed by Lempinen (1964) to cause a pronounced hyperplasia of the extra-adrenal chromaffin tissue, which normally degenerates within 2 weeks after birth He used fixation in the mixture of potassium dichromate and formalin for the demonstration of catecholamines in the chromaffin cells With the aid of this reaction supplemented with the Schmorl reaction, Lempinen (1964) found numerous reactive cells also in the sympathetic ganglia of the young rats treated with cortisone or hydrocortisone, although no or few chromaffin cells were observed in the sympathetic ganglia of the untreated rats

While the effect of adrenal cortical steroids on the chromaffin tissue has been confirmed chemically by paper chromatography (Eranko, Lempinen and Raisanen 1966)

and by quantitative catecholamine determinations (Roffi and Margolis 1966 see also the recent review by Pohorecki and Wurtman 1971), the appearance of small chromaffin cells in the sympathetic ganglia has not been investigated since However such a study is of obvious interest, because small cells have been observed even in normal sympathetic ganglia which exhibit an intense formaldehyde induced fluorescence due to catecholamines (Eranko and Harkonen 1963, 1965) These small intensely fluorescent (SIF) cells, although they do not give the chromaffin reaction have been observed to possess structural features similar to those of the truly chromaffin cells (Matthews and Raisman 1969, Eranko and Eranko 1971a)

The present study, in which catecholamines have been studied in the sympathetic ganglia and the extra-adrenal chromaffin tissue with the aid of formaldehyde induced fluorescence, shows that hydrocortisone has indeed dramatic effects on the SIF cells of the sympathetic ganglia

Material and Methods

Experimental

Newborn rats were injected i p with hydrocortisone daily for 5 days Hydrocortisone acetate (kindly supplied by N V Organon) was dissolved immediately before injection in 0 9 % sodium chloride solution to make a concentration of 4 mg/ml The daily dose was 40 mg/kg of b w i e 0 4 mg to a 10 g rat They were killed by cutting the back at the level of the heart on the 6th day after birth together with untreated litter mate controls While the weight of the controls increased from about 10 g at birth to about 12 g on the 16th day the weight of the injected rats which all had a diarrhea decreased from about 10 to 8 g Two litters, each divided into 6 experimental and 6 control animals were examined, or together 12 injected rats and 12 controls

Adult male rats weighing about 200 g were injected i p with 40 mg per kg of b w of hydrocortisone acetate daily for 14 days using the same solution as for the young rats They were killed on the 15th day The weight of the injected animals decreased during the treatment from 200 g to about 190 g while that of the controls increased to about 250 g The adrenal weight of the injected animals decreased to about one half of that of the controls 10 injected rats and 10 controls were studied

Demonstration of catecholamines

Immediately after killing the superior cervical ganglia and in the young rats, the retroperitoneal tissue block between the kidneys and the adrenals over the vertebral column were removed placed on an aluminium foil and frozen by immersion in propane cooled to — 190° C with liquid nitrogen Thereafter they were freeze dried for 1 week at — 45° C in vacuum warmed removed from the freeze drying apparatus and exposed to formaldehyde vapour generated from paraformaldehyde powder equilibriated with 60 % relative air humidity for details see Eranko (1967) After exposure for 30 min at 50° C and 1 hour at 80° C to formaldehyde the tissues were embedded in a mixture of Epon and Araldite (Eranko and [illegible] ...es of sections [illegible] ...arme... [illegible] ...eitz, [illegible] ...Gen Mainz) after the Osram HBO 200 lamp 3 mm BG 38 3 mm BG 3 T AL 405 K 470

Fig 1 Formaldehyde induced fluorescence in the retroperitoneal tissue block of a 6-day-old control rat A aorta V vena cava LG lumbar sympathetic ganglion, OZ, Organ of Zuckerkandl ×60

Fig 2 Similar field as Fig 1 of a 6 day old rat injected daily after birth with hydrocortisone Note the numerous fluorescent cells in the lumbar ganglion (LG) and the very intense fluorescence of the Organ of Zuckerkandl (OZ) ×60

Fig 3 Lumbar ganglia of a rat injected with hydrocortisone The ganglia contain numerous fluorescent cells ×120

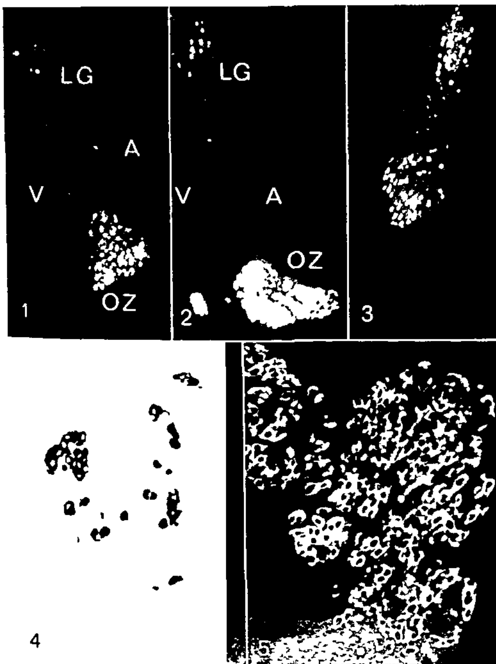


Fig 4 The Organ of Zuckerkandl of a control rat The photomicrograph is underexposed to comply with the very intense fluorescence of the small cells The larger weakly fluorescent cells (see Fig 1) are therefore hardly visible × 250

Fig 5 The Organ of Zuckerkandl of a hydrocortisone injected rat Conditions of photography as in Fig 5 The body is mainly composed of small intensely fluorescent cells

Results

A cross section of the retroperitoneal tissue block of a normal young (6 day old) control rat is illustrated in Fig 1 A weak formaldehyde induced fluorescence is visible in the lumbar ganglion (LG), in which 2 somewhat more intensely fluorescent cells are also seen The main para-aortic body, the organ of Zuckerkandl (OZ) exhibited a more intense fluorescence than that in the ganglion, mainly because of the presence of small intensely fluorescent cells scattered amongst less intensely fluorescent cells which were more concentrated in the central end of the body This distribution of the cells exhibiting monoamine fluorescence closely corresponded to the distribution of chromaffin cells demonstrated by fixation in dichromate formalin mixture in the study by Lempinen (1964), as is evident by comparing his Fig 34 with our Fig 1 Fig 4 is a fluorescence photomicrograph taken at a higher magnification of the OZ of another control rat Clusters of intensely fluorescent cells are seen against the background of less fluorescent cells

The retroperitoneal tissues of a 6 day-old rat injected daily with hydrocortisone injections since birth are illustrated in Fig 2 made under similar conditions of preparation and photomicrography as Fig 1 Two main differences can be seen, which were typical of all the young hydrocortisone-treated rats examined Firstly, the num



Fig 6 Amine fluorescence in the superior cervical ganglion of a control rat Note the 2 moderately fluorescent cells marked with arrows amongst relatively weakly fluorescent young sympathetic cells and the small intensely fluorescent cells (SIF) ×250

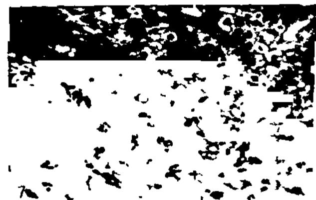


Fig 7 Similar fluorescence photograph of the same ganglion of a rat injected with hydrocortisone There are numerous SIF cells all over the field ×250

ber of SIF cells in the sympathetic ganglia, also shown in Fig 3, was much increased as compared with the controls Secondly, the whole OZ was filled with cells whose fluorescence intensity appeared to be even more intense than that in similar cells observed in the control rats These cells are better shown in Fig 4 and 5, which have been exposed less than Fig 1—3 to avoid "washing out" of the details due to the intense fluorescence as in Fig 2

The monoamine fluorescence of the superior cervical ganglion of a young control rat is illustrated in Fig 6 The ganglion cells were at the age of 6 days still small and closely packed because of the relatively small amount of structures such as dendrites axons and Schwann cells between them The intensity of the cytoplasmic fluorescence varied considerably between individual ganglion cells Most cells exhibited a weak, though clearly discernible fluorescence, which was located especially around the nucleus About one tenth of all the cells, 2 of which are indicated by arrows in Fig 6, exhibited a moderate fluorescence Further cells, which were rare, showed a fairly intense fluorescence They were therefore considered SIF cells However, it should be pointed out that the fluorescence of these cells was less intense than that in the SIF cells in the sympathetic ganglia of normal adult rats and very much less intensely fluorescent than the cells of the OZ or adrenal medulla

After treatment for 5 days with hydrocortisone, a dramatic increase in the number of SIF cells was observed in the superior cervical ganglion of young rats as can be seen in Fig 7 Comparison with Fig 6 shows that while most young ganglion cells had remained similar to those in the control ganglion numerous clusters of SIF cells appeared everywhere in the ganglion To obtain an idea of the order of magnitude of the increase in the number of SIF cells, these were counted from a complete series of sections from one control ganglion and one ganglion from a hydrocortisone treated rat In the control ganglion 328 SIF cells were found while 3110 cells were found after treatment with hydrocortisone Although no further cell counts were made, because these were so tedious owing to the large number of evenly scattered cells, visual examination of the complete series of sections in all control and experimental animals clearly showed that the increase in the number of SIF cells was of the same order in all young injected rats

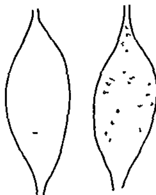


Fig 8 Schematic drawing showing the distribution of SIF cells in typical mid sections of the superior cervical ganglia of two 6 day-old rats On the left the ganglion of an untreated control rat with 25 SIF cells On the right the ganglion of a rat injected since birth daily with hydrocortisone there are 259 SIF cells in the mid section Note the even distribution of the newly formed SIF cells after hydrocortisone treatment

The distribution patterns of the SIF cells were also studied It proved useless to draw a composite picture of the distribution of SIF cells in the ganglion of young hydrocortisone injected rats from a complete series of sections, the whole ganglion being covered by the SIF cells but comparison of the midsections drawn in Fig 8 clearly illustrate the effect of hydrocortisone New SIF cells covered the whole ganglion in a single mid section while only some areas show SIF cells in the mid section of the control ganglion It is therefore probable that new SIF cells had been formed from less intensely fluorescent cells Cells with a moderate cytoplasmic fluorescence such as the two cells marked with arrows in Fig 6 were seen everywhere even in the normal sympathetic ganglion of young rats and thus can be considered as cells potentially capable of developing into SIF cells However, it is likely that other even less fluorescent and less differentiated cells may serve as stem cells for the SIF cells which were formed under the influence of hydrocortisone

Preliminary electron microscopic study of the superior cervical ganglion of 6-day old rats treated with hydrocortisone known from fluorescence microscopy to contain large numbers of SIF cells failed to demonstrate cells with numerous small granular vesicles typical of the SIF cells of adult animals No such cells were found after examination areas large enough to contain several SIF cells All cells showed electron microscopic signs of intense cytoplasmic activity the nuclear membrane was fenestrated the endoplasmic reticulum the Golgi apparatus and the mitochondria were well developed and numerous Although some granular vesicles of about 50 nm in diameter were seen in the axons between the cell bodies granular vesicles were extremely rare in the cytoplasm of the young ganglion cells Further studies are required to characterize the fine structure of the SIF cells formed after hydrocortisone treatment

There was no difference discernible by fluorescence microscopy in the appearances of the superior cervical ganglion of the adult hydrocortisone treated rats and those of the controls The distribution and number of the SIF cells remained essentially unaffected In some injected animals there were clusters with increased numbers of SIF cells but the changes were not significant Other ganglia or para aortic tissues were not studied in the adult rat

Discussion

The observation made in the present study that administration of hydrocortisone in newborn animals causes a hyperplasia of the extra adrenal chromaffin tissue and the appearance of numerous SIF cells in the sympathetic ganglia confirm with fluorescence microscopical techniques the observations made by Lempinen (1964) who used the Schmorl reaction after dichromate fixation to demonstrate catecholamine containing cells As was also noted in his study the effect of hydrocortisone depended on a sufficiently early beginning of the administration of cortical hormone which had no essential effect on the adult sympathetic ganglia

The effect of some other drugs causing alterations in the sympathetic ganglia also depend on beginning the administration before the animals are too old Thus in

jections of antiserum against the nerve growth factor almost totally destroy almost all sympathetic nerve cells when given to a newborn mouse or rat but have little effect on the sympathetic ganglion of adult animals (Levi Montalcini and Angeletti 1966) The same applies to "chemical sympathectomy" with 6-hydroxydopamine (Angeletti and Levi Montalcini 1970) or with guanethidine (Eränko and Eränkö 1971b, c) *It appears plausible that the degree of differentiation of the ganglion cells* increases resistance against the destructive mechanisms

On the other hand it has been observed that guanethidine, which destroys young nerve cells, causes a proliferation of the SIF cells in the sympathetic ganglia of newborn rats, as does hydrocortisone, but has no such effect in adult rats, again like hydrocortisone (Eränkö and Eränko 1971 c) However, their mechanisms of action resulting in the SIF cell proliferation need not be the same. Indeed, hydrocortisone and guanethidine cause different types of SIF cell increase While hydrocortisone administration results in a wide spread new formation of SIF cells throughout the ganglion, guanethidine causes an increase in the number of SIF cells within the clusters of SIF cells which are normally present in untreated rats of the same age (Eränkö and Eränkö 1971 c) It seems that hydrocortisone has a true inductive action leading to the formation of SIF cells from previously undifferentiated stem cells which have still a potency to become a sympathicoblast or a phaeochromoblast (or SIF cell stem cells) This view was also presented by Lempinen (1964) On the other hand guanethidine appears to cause the proliferation of already differentiated SIF cells

Some caution is necessary in drawing this conclusion, because the rats of the present study were killed immediately after cessation of the hydrocortisone treatment, at the age of 6 days while the guanethidine treated rats were permitted to recover for 3 weeks after 8 daily drug injections and killed at the age of one month (Eränko and Eranko 1971 c) The formation of clusters of SIF cells is more advanced in one month-old normal rats than in 6-day-old rats While further studies are therefore desirable, it seems from Lempinen s (1964) study that the small catecholamine containing cells formed because of cortisone administration remain scattered in the ganglion without formation of clusters (see his Fig 00 of the ganglion of a two-month old rat)

It should also be emphasized that the SIF cell is a descriptive term of wide content Therefore, there may be SIF cells whose other properties are different although they share the small size and the intense fluorescence characterizing the SIF cells Our preliminary electron microscopic observation failed to provide evidence for the expected presence of small granular vesicles in the cytoplasm of the SIF cells newly formed after hydrocortisone treatment, while granular vesicles similar to those of the chromaffin cells are typical features of adult SIF cells (Eranko and Harkonen 1965 Matthews and Raisman 1969) Such vesicles caused Siegrist *et al* (1968) to call these cells chromaffin cells, although in the rat they are non chromaffin *i e* they do not become coloured after dichromate fixation alone The presence a high concentration of catecholamine in the cytoplasm of the new SIF cells which is beyond

reasonable doubt proved by the intense formaldehyde induced fluorescence in spite of the absence of electron microscopically demonstrable granular vesicles, suggests that high concentrations of catecholamines may be present in a cytoplasmic pool outside special storage organelles like the granular vesicles Such a diffuse pool was proposed by Eranko and Harkonen (1963) on the basis of fluorescence microscopic appearances of the ganglion cells of the superior cervical ganglion, the fluorescence being in part evenly distributed over the cytoplasm, in part localized in granules. Aggregates of granular vesicles were recently electron microscopically demonstrated by Van Orden *et al* (1970) in the region of the fluorescent granules of the sympathetic ganglion cells Our preliminary observations (unpublished) on normal adult ganglion cells suggest that the catecholamine stores exhibiting a diffuse perinuclear fluorescence correspond to the endoplasmic reticulum

Even if further investigations are required to make clear the properties of the different types of SIF cells, their proliferation and new formation after administration of steroids such as hydrocortisone is an interesting phenomenon, which offers new possibilities for understanding the nature of the SIF cells Moreover, it raises interesting new questions of the role of adrenocortical hormones in the normal differentiation and function of sympathetic and other nerve cells

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From the Institute of Physiology, University of Lund, Sweden

The Motility of the Pancreatic Duct of the Cat; an in-vitro Study

by

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Abstract

LENNINGER S *The motility of the pancreatic duct of the cat, an in vitro study* Acta physiol scand 1972 *84* 134—141

Segments of the pancreatic duct of cats were suspended in Krebs solution and connected to a force-displacement transducer Spontaneous, rhythmic contractions were recorded from all parts of the duct The contractions seem to be exerted by longitudinally orientated muscle cells and are probably myogenic Acetylcholine bradykinin and histamine increased the tension. Noradrenaline caused a slight increase in tone while isoprenaline relaxed the duct The spontaneous activity was annulled when bile was present in the bath

The flow of juice from the pancreas into the intestinal lumen is dependent both on the secretory activity of the glandular cells and on the status of the excretory ducts of the gland When the duodenal orifice of the large pancreatic duct is inspected through a duodenal fistula the juice can be seen to flow discontinuously into the gut This irregularity of the flow may readily be ascribed to the influence of the periodic motor activity of the duodenal wall through which the pancreatic duct pierces It has also been proposed however, that the duct has contractile ability by itself and that the duct may affect the flow from the gland (Anrep 1914 Korovitsky 1923 Babkin 1924 Scratcherd 1970) The present experiments confirm that the pancreatic duct of the cat has contractile properties

Methods

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[illegible] embolus the pancreatic [illegible] moving most of the [illegible] e gland When the [illegible] n (see below) and [illegible]

kept refrigerated during the night The final preparation of the segments was performed under dissection microscope with the duct immersed in Krebs solution at room temperature Remaining glandular tissue was removed and the duct was divided into a number of segments, each about 7 mm long Silk ligatures (6/0) were tied in both ends In 6 of the experiments the mutual order of the segments was recorded in order to compare different parts of the duct. In the other experiments studies were usually performed on segments taken about half way between the splenic and duodenal ends of the duct

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silk ligatures the segments were connected to a force displacement transducer (Grass F T 03) operating an inkwriting polygraph (Grass) A passive tension of approximately 500 dyn was applied to the segments

In an attempt to detect contractions caused by circularly arranged muscle cells 6 of the segments were cut open in a helical fashion before they were mounted 3 of these segments were [illegible] approximate angel of 45°
[illegible] choline chloride atropine sulphate isoprenaline
[illegible] lol hydrochloride papaverine sulphate histamine
[illegible] supplied by Sandoz) secretin and cholecystokinin
[illegible] Institutet Stockholm) The concentrations of secretin and CCK are expressed in clinical units/ml and Ivy dog units/ml respectively The concentrations of the other substances are expressed in g/ml and refer to the compounds as given above with the exception for noradrenaline which is calculated as base

Results

Spontanous activity 86 of the 91 tubular segments and 3 of the 6 helical strips developed active tension and showed spontaneous rhythmic contractions The activity started within 15 min after mounting the preparations in the bath and then continued for several hours The activity of the helical strips was small compared to that of the tubular segments If a previously briskly contracting segment was transformed to a helical strip it lost almost all its activity Cutting a segment longitudinally, on the other hand, affected its contractility only to a small extent

The basal active tension varied between 10 and 200 dyn in different segments as calculated from the spontaneous rise of the baseline from its lowest position during a phase of relaxation (Fig 1) Spontaneous periodic activity was superimposed on the basal tone It consisted of phasic contractions which either occurred randomly and were of varying strength or were regular both in rhythm and strength (Fig 2) Intervals of up to 1 min were registered between the regular contractions A segment was often seen to change its pattern of activity from an irregular to a regular rhythm or vice versa during the course of the experiments

Considerable difference in motility was seen in different segments of the same duct Thus while only small oscillations were produced by some segments others exerted strong regular contractions It could not be found however that segments from one particular part of the duct regularly were more active than segments from some other part The highest tension observed during a spontaneous contraction was 800 dyn This corresponds to about 0 27 kg/cm cross sectional area as the weight of the segments was 3 mg and its length in the bath approximately 1 cm

The tone and spontaneous contractions were not affected by atropine in concentrations up to 10^{-5} but were abolished by papaverine in a concentration of 10^{-6}



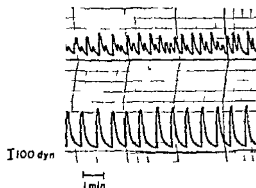


Fig 1 Fig 2

Fig 1 Spontaneous rise of tonus after a period of relaxation

Fig 2 Spontaneous motility of two segments from the same duct

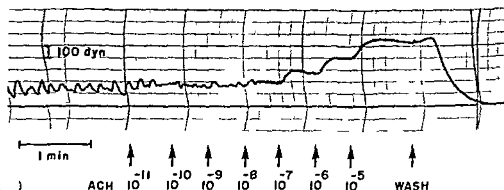
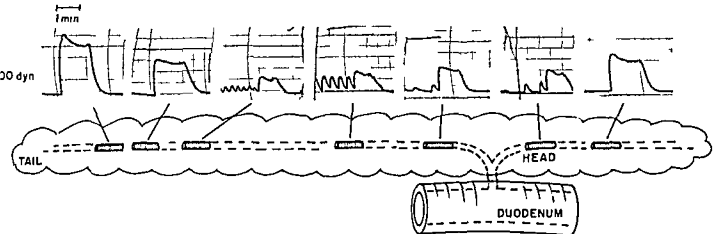


Fig 3 Response to increasing concentrations of acetylcholine

Effects of acetylcholine Acetylcholine caused an increase in tension in all the segments which had showed spontaneous activity The responses of the helical strips were much smaller than those of the tubular segments No further experiments were therefore performed with the helical strips

The smallest dose to increase tension was determined in 30 segments from 12 cats by giving acetylcholine in increasing amounts A rise in the baseline was seen with concentrations between 10^{-11} and 10^{-6} (median 10^{-8}) g/ml By increasing the concentration the active tension was further increased until a sustained smooth tetanus or contracture ensued A typical response is shown in Fig 3 The response to a submaximal concentration started a few seconds after the acetylcholine had been added to the bath and full response was attained within 30 s The tension then declined slightly but was fairly well maintained as long as the acetylcholine was present in the bath After removing the acetylcholine the tension decreased rapidly and was for some time lower than prior to the stimulation The responses of a segment to repeated similar concentrations of acetylcholine were almost identical



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Fig 1

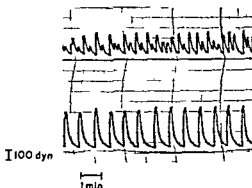


Fig 2

Fig 1 Spontaneous rise of tonus after a period of relaxation

Fig 2 Spontaneous motility of two segments from the same duct

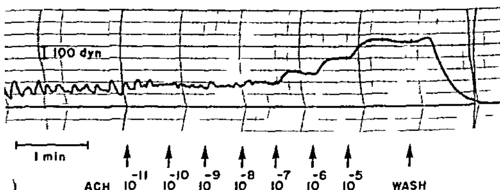


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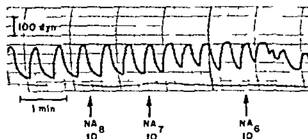


Fig 6 The response to increasing concentrations of noradrenaline

amounts was also given to 7 segments from 5 cats after propranolol (10^{-6} g/ml) had been added to the bath In all segments a small but consistent increase in tone could be seen when the concentration of noradrenaline was successively increased from 10^{-8} to 10^{-6} g/ml A higher concentration occasionally decreased the tone, however (Fig 6)

Effects of bradykinin and histamine Eight segments from 5 cats were exposed to bradykinin in concentrations between 10^{-11} and 10^{-6} g/ml Four segments from 2 cats responded with an increase in tension at concentrations of 10^{-9} and 10^{-8} g/ml respectively The responses were smaller than those obtained with acetylcholine in similar concentrations

Histamine was tested in 3 segments from 3 cats At a concentration of 10^{-6} g/ml an increase in tone was seen Also the responses to histamine were smaller than with similar concentrations of acetylcholine

Effects of gastro intestinal hormones 6 segments from 3 cats were exposed to secretin and 10 segments from 6 cats to CCK in concentrations between 0 001 and 0 1 units/ml No effect on tone or rhythm was seen

Effects of pancreatic juice and bile In 2 expts with 4 segments 0 5 ml of pancreatic juice, collected from cats which had been given secretin and methacholine intravenously was added to the bath It did not affect the tension or rhythm of the segments The segments were also exposed to the same amount of bile drawn from the gall bladder of cats It promptly lowered the tone and the rhythmic contractions of the segments disappeared

Discussion

Data regarding the fine structure of the pancreatic duct is scarce in the literature and the presence of contractile elements in the ductal wall is only occasionally mentioned Eberth (1863) has briefly described a layer of longitudinal muscles in the pancreatic duct of the cat In some later investigations with light and electrone microscopes (Richins 1945 Coupland 1958, Watari 1968) smooth muscle cells in the pancreatic duct of the cat are noticed but no close description as to their number or arrangement is given In a recent investigation, however, with particular interest paid to the occurrence of smooth muscles in the ductal wall of the cat s pancreas, a lining of

muscle cells was found along the duct from the tail of the gland to its duodenal end (Garrett 1970) This finding agrees with the present observation that motility can be recorded from practically all parts of the pancreatic duct The occurrence of syn chronized spontaneous contractions requires a close contact between the muscle cells and this finding therefore indicates that a continuous layer of smooth muscle is present in the duct It is likely, however, that the cells are unevenely distributed along the duct as the contractility varied from one part of the duct to another The muscle cells seem to be orientated mainly parallel to the length axis of the duct as judged from the high active forces developed by the longitudinal preparations The weak and inconsistently found active tension in the helical strips, on the other hand, speaks against the presence of a circular muscle layer

The motor activity of the pancreatic duct of the cat shows great similarity to that of an intestinal preparation Thus, it is spontaneously rhythmic and is stimulated by acetylcholine, histamine and bradykinin In sensitivity to acetylcholine the duct compares well to guinea pig taenia coli (Schatzmann 1968) The responses to cate cholamines, on the other hand, show some interesting features The characteristic response of intestinal smooth muscle to these agents is relaxation (Schatzmann 1968) In some preparations, however, such as the longitudinal smooth muscle of the esophagus of the cat relaxation is brought about by isoprenaline while noradreanline causes contraction (Christensen and Daniel 1966) The cat's pancreatic duct reacts in a similar way Thus it seems to be provided with receptors of the β type causing the muscle cells to relax and with α receptors causing the muscle cells to contract

The spontaneous rhythmic activity of the duct is most likely myogenic This sumption is based on the fact that the activity persisted after more than 24 h anoxic, cold storage and also on the finding that it was not inhibited by atropine n vivo however the motility is probably modified by nervous impulses Histological studies have revealed that cholinergic nerves are present in the vicinity of the duct (Richins 1945 Watari 1967) and the present studies show that receptors for both acetylcholine and catecholamines are associated with the contractile elements of the duct

The flow of juice from the cannulated pancreatic duct or of a salt solution perfused through the duct is often retarded by cholinergic stimulation (Anrep 1914, Korovitsky 1923 Scratcherd 1970 Lenninger 1971) This finding has been taken as evidence that the stimulation causes contraction of the duct and constriction of its lumen (Anrep 1914 Korovitsky 1923 Scratcherd 1970) Although the present findings confirm that contractions may take place in the pancreatic duct they do not explain how the supposed constriction of the lumen is brought about Thus it is not obvious how contraction of longitudinal muscle layer can cause narrowing of the ductal lumen It is also unknown what may be the physiological significance of the contractility of the pancreatic duct It is possible that the duct takes part in the propulsion of juice It may also be speculated that the contractions affect the openings of the small side branches which empty into the large duct and so regulate the flow between the parenchymatous part of the gland and the excretory duct Finally,

the longitudinal muscle layer may help to keep the duct straight similarly to what has been discussed for the longitudinal muscles of the portal vein (Johansson 1970) Such a function might be relevant considering the fact that the pancreas of the cat lies intraperitoneally and is only losely attached to neighbouring organs

The gastro-intestinal hormones had no influence on the motility This was hardly to be expected for secretin but might have been anticipated with CCK which affects the motility of other parts of the gastro-intestinal tract Also diluted pancreatic juice was without effects on the motility while bile promptly inhibited the spontaneous motility The inhibitory effect of bile has been demonstrated on other preparations of smooth gastro-intenstinal muscle (Schüpbach 1908) and is attributed to its content of bile salts (D Errico 1910)

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Supraspinal Control of Monosynaptically Activated Group Ia Interneurones in the Ventral Horn

By

H Hultborn and M Santini

Disynaptic reciprocal Ia inhibition is facilitated from several descending pathways (the corticospinal tract, Lundberg and Voorhoeve 1961, the rubrospinal tract, Hongo, Jankowska and Lundberg 1969, the vestibulospinal tract, Grillner, Hongo and Lund 1966) In all these studies spatial facilitation was used as a tool—an excitatory convergence from different neuronal systems on the Ia inhibitory interneurones being deduced from the facilitatory actions on Ia IPSPs (inhibitory post synaptic potential) recorded in motoneurones Hultborn, Jankowska and Lindström (1971) identified interneurones in the ventral horn with a convergence of monosynaptic excitation and inhibition from motor axon collaterals and they postulated that these interneurones mediate the reciprocal Ia inhibition to motoneurones In fact interneurones identified with their criteria were recently shown to produce onosynaptic IPSPs in motoneurones (Jankowska and Roberts 1971) In the present vestigation we have tested whether these Ia coupled interneurones in the ventral horn are subject to the descending actions postulated for the interneurones mediating reciprocal Ia inhibition (Lundberg and Voorhoeve 1961, Hongo *et al* 1969, Grillner *et al* 1966)

Two series of experiments were performed one series in which the red nucleus (NR) and pyramid (Pyr) were stimulated (6 cats) and another series (7 cats) in which the Deiters nucleus (ND) was stimulated No difference in action on the Ia coupled interneurones could be detected between the stimulation of the pyramid in the first series and stimulation of the hindlimb area of the sensorimotor cortex in two control experiments The dissection maintenance of the preparation stimulating and recording system as well as location and stimulation of supraspinal centres has been described elsewhere (*Hultborn and Udo 1972 a b*) The interneurones were identified by their monosynaptic Ia excitation inhibition by impulses in motor axon collaterals absence of antidromic invasion from ventral roots or spinal halves (at Th 12 level) and finally their ability to follow orthodromic stimulation up to 300—500/s (see Hultborn *et al* 1971) The material consists of 87 such interneurones located in the ventral horn and connected with different hindlimb muscle nerves 28 of them were impaled and recorded intracellularly

Fig 1 illustrates pyramidal and rubrospinal effects on an interneurone which is monosynaptically excited from the adductor (Add) nerve The recurrent inhibition, as indicated by the obliteration of its spike potentials, is illustrated in records B—C and its ability to follow stimulation frequencies of 100/s in D The test stimulus alone (Add 1 2×thresh) was not enough to elicit the spike (E) but the same test stimulus

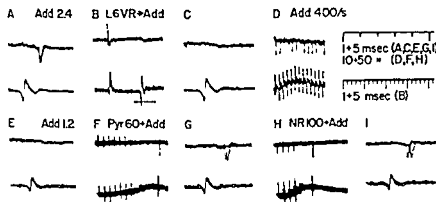


Fig 1 *Pyramidal and rubrospinal excitation of a Ia coupled interneurone* Upper traces are extracellular records from an interneurone Lower traces are cord dorsum potentials Dashed lines below [illegible]

[illegible] fibres Strength of stimulation of the pyramid and the red nucleus is given in μA

evoked a discharge when preceded by conditioning stimulation of the pyramid (F—G) or the red nucleus (H—I), thus showing the pyramidal and rubrospinal excitation of this interneurone Pyramidal or rubrospinal volleys could usually by themselves evoke discharges of these Ia coupled interneurones although higher stimulus strengths and/or longer trains of stimuli to the supraspinal structure were then needed These pyramidal and rubrospinal effects were similar for extensor as well as flexor coupled interneurones

During intracellular recording current passage through the recording microelectrode was used to analyze a possible convergence of excitatory and inhibitory actions and it was found that the pyramidal and rubrospinal systems did not merely evoke EPSPs (excitatory postsynaptic potentials), but a mixture of polysynaptic EPSPs and IPSPs Fig 2 A—E illustrates one of 3 quadriceps-coupled interneurones in which also a short latency rubrospinal IPSP was disclosed The monosynaptic Ia excitation from quadriceps (Q) and the recurrent inhibition from L5 ventral root are shown in records A—B Records in C—E illustrate the responses to a single and double shock to the red nucleus The second shock evoked an IPSP with a segmental latency of 1 5 ms (as measured from the second descending rubrospinal volley marked by the arrow in E, *cf* Hongo *et al* 1969) thus indicating a disynaptic linkage

Records F—I in Fig 2 illustrate the common finding that the vestibulospinal tract evokes a *monosynaptic excitation of* Q excited Ia interneurones (which are probably inhibiting posterior biceps semitendinosus (PBSt) motoneurones) On the contrary PBSt coupled interneurones (probably inhibiting Q motoneurones) never received any short latency facilitation from the vestibulospinal tract This pattern

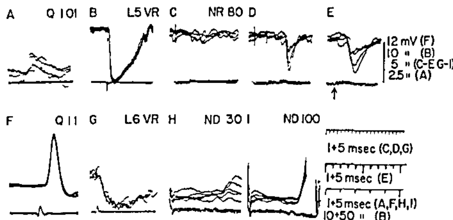


Fig 2 *Rubrospinal and vestibulospinal connexions* A—E illustrate a disynaptic rubrospinal IPSP *in an interneurone with Ia connexions from Q F—I show a monosynaptic EPSP from* the vestibulospinal tract in another Q motoneurone Upper traces are intracellular recordings and the lower traces cord dorsum potentials The dashed line below records in D show the part expanded in E The arrival of the effective descending rubrospinal volley is marked by the arrow in E Strength of stimulation of the red nucleus and the Deiters' nucleus is given in μA

of vestibulospinal effects on Ia excited interneurones shall be compared with the vestibulospinal facilitation of Ia IPSPs in PBSt motoneurones from the Q nerve and lack of any correspondent facilitation in the reverse direction (Grillner *et al* 1966)

To summarize, stimulation of defined supraspinal systems has shown that the Ia coupled interneurones in the ventral horn are subject to the descending actions stulated for the interneurones mediating reciprocal Ia inhibition (Lundberg and Voorhoeve 1961, Hongo *et al* 1969, Grillner *et al* 1966) Furthermore it was revealed that cortico- and rubrospinal volleys have not a purely excitatory action, but in fact evoke a mixture of EPSPs and IPSPs In case of the rubrospinal inhibition the minimum linkage was disynaptic

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A Respirometer for Tissue Cultures of Liver

By

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Abstract

SANDSTRÖM, B *A respirometer for tissue cultures of liver* Acta physiol scand 1972 *84* 145—150

A simple differential respirometer equipment for experimental work with tissue cultures of liver is described The respirometer works with small amounts of tissue (0 3—3 mg dry weight) The respiration values obtained indicate that the employed cultivation technique permits gas exchange at a rate adequate for normal liver also when the working gas phase consists of ordinary air

The oxygen consumption is an essential parameter in experimental work with tissue and organ cultures Several respirometers have been constructed to fit various culture techniques However, none of these have been successful with respect to culture of functionally representative liver

Recently Lucas (1965) has constructed a differential respirometer for the work with Trowell's organ culture technique (1959) This technique permits successful culture of fragments from several mammalian organs but is unfortunately not suited for liver (Trowell 1961)

At present the most successful technique for maintaining functionally active liver tissue for experimental purposes in culture appears to be the method of Sandstrom (1964, 1966) and modifications thereof (Alexander and Grisham 1970)

In order to make possible measurements on the oxygen consumption of liver cultures, the culture chamber has been modified for the use with a simple differential respirometer device These arrangements will be described in the present communication

Description of apparatus and technique

Respirometer In a rectangular block of perspex (20 cm × 10 5 cm × 6 cm) two cylindrical wells (51 mm deep, 81 mm in diam.) are milled out *Each of these wells* is made to contain four culture chambers according to the dimensions given in the

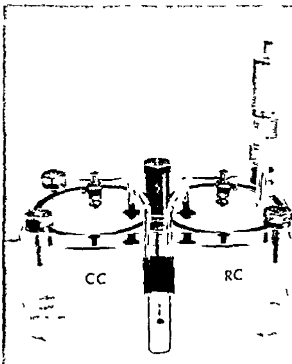


Fig 1 Front view of the respirometer For closer description see text CC, compensation chamber RC, reaction chamber

original description (Sandstrom 1964) and they constitute the reaction chamber (RC in Fig 1) and the compensation chamber (CC) of the differential respirometer The chambers are closed with a rectangular lid of perspex (20 cm × 10 5 cm × 1 cm) screwed onto the perspex block with 6 bolts (Fig 1) To get a perfect tightening rubber O'-rings are placed in a circular moat around each chamber opening Over each chamber is made a hole in the lid to fit a Gilmont differential syringe manometer W 4200 To get a higher degree of precision the chambers may be connected with a 0 3—0 5 mm—bore borosilicate-glass tubing bent according to Fig 1 and fixed in the lid with rubber cement Over each chamber must then be a 2 way stopcock for gassing and equilibration and over the reaction chamber there must be a hole to fit a Gilmont micrometer syringe S 3200 or S 1100 To make the working gas volume as small as possible all empty space in the reaction and compensation chambers is filled with pieces of perspex In this way the working gas volume of each chamber is brought to about 38 ml with a maximum difference of 4 ml between the chambers

The manometer fluid consists of Shell® kerosene (paraffin oil) colored with Scarlet red (Sudan IV) It has a specific gravity slightly less than 0 78

Temperature control To keep temperature variations at a minimum the respirometer (except the upper part of the micrometer syringe) is immersed in a 100 l water-bath with a temperature of +37° C and temperature variations within

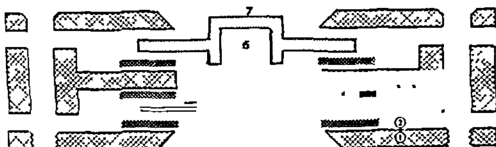


Fig 2 Longitudinal cross-section through a disassembled culture chamber 1 bottom plate of stainless steel This plate is srewed together with the middle and the top plate by means of screws through the vertical holes at the left and the right ends of the plates 2 rubber tightening for the cover glass (3) 4 ring of foam plastic for the culture medium, 5, sandwich of perforated and unperforated cellophane discs with a piece of liver tissue between, 6 pad of foam plastic for the carbon dioxide absorbent, 7, perspex plate with central circular cup (Dimensions approximately double natural size)

±0 01° C To avoid that cool air comes into the chambers at equilibrations, the stopcocks over the reaction and the compensation chambers are connected by means of plastic tubing to an air reservoir immersed in the water bath

Culture chamber To get effective absorbtion of the liberated carbon dioxide the culture chamber (Sandstrom 1964) is provided with a small reservoir for the carbon dioxide buffer Thus the cover glass and its rubber tightening opposite the tissue explant are replaced by a circular 1 mm thick perspex plate with a small cup, 10 mm wide and 4 mm deep milled out in the centre (Fig 2 and 3) Those perspex plates are sterilized in formalin vapour, since they cannot be autoclaved or boiled Into the small cup in the centre a piece of foam plastic is placed to be moistened with the carbon dioxide absorbent, which may be dropped onto the foam plastic by means of a thin hypodermic needle inserted through one of the side channels in the same manner as for feeding of the cultures To avoid contact between the carbon dioxide absorbent and the outer ring of foam plastic containing the culture medium the

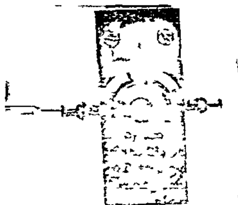


Fig 3 A culture chamber seen from above A hypodermic needle is inserted through one of the side holes The tip of the needle is just above the pad of foam plastic for the carbon dioxide absorbent The horseshoe-shaped foam plastic ring for the culture medium is seen at the periphery

central cup has a small rim projecting towards the tissue explant During the experiments the culture chambers are always positioned with the tissue explant upwards since carbon dioxide is heavier than oxygen

Carbon dioxide absorbent The carbon dioxide may be absorbed by potassium hydroxide For tissue culture purposes a 2 % solution, that is nearly isotonic is used However, alkali can only be used when the working gas phase consists of ordinary air and the culture medium is not carbonate buffered When the working gas phase should contain a certain percent carbon dioxide in air or oxygen, a diethanolamine buffer is employed as described by Lucas (1965)

Respirometer procedure Liver cultures are set up and maintained as described previously (Sandstrom 1964 1966) At appropriate time during the culture course the foam plastic in the central cup (Fig 2/6) opposite the liver explant is soaked with carbon dioxide absorbent which is dropped onto it by means of a hypodermic needle inserted through one of the side holes in the culture chamber The side holes are left open and four culture chambers are placed in the reaction chamber of the respirometer In the compensation chamber are placed four sham cultures that contain no liver explants but otherwise have been similarly treated The respirometer lid is screwed into place and the respirometer immersed in the water bath When a working gas phase other than air is used the chambers are gassed for 10 min with the appropriate gas mixture through a thin and long hypodermic needle inserted through the stopcocks over the respirometer chambers

After 2 h temperature equilibration the stopcocks over the reaction and compensation chambers are opened simultaneously to equilibrate with athmospheric pressure the micrometer syringe is screwed into zero position and after that the stopcocks are closed

When the liver cells consume oxygen a pressure difference is produced between the reaction and compensation chambers observable on the different fluid levels in the legs of the manometer At regular intervals this difference is equilibrated by means of the micrometer syringe the plunger of which is screwed downwards This volume corresponds to the amount of consumed oxygen Correction to standard temperature and pressure (0° C 760 mm Hg) is made as usual

Results

Our experience with this respirometer is so far restricted to embryonic chicken liver which is possible to maintain functionally active in culture (Sandstrom 1966) Some successful preliminary experiments with adult human liver (needle biopsies) have also been carried out (Sandstrom 1971) In most instances the working gas phase has been air and either diethanolamine or potassium hydroxide has been used as carbon dioxide absorbent For 15 day old chicken liver cultured for 1 to 5 days a linear oxygen consumption of 12 ± 1 µl/h and mg dry weight Q_{O_2} = 12 ± 1) has regularly been observed with potassium hydroxide as carbon dioxide absorbent The values are

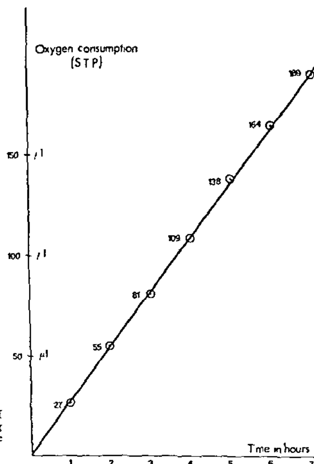


Fig 4 Oxygen consumption of 15 day old chicken liver (2 25 mg dry weight) during 7 h on the fifth day in culture

about 20 % lower when diethanolamine carbon dioxide buffer is employed A typical example is shown in Fig 4 referring to the oxygen consumption of 15 day-old chicken liver, in culture for 5 days over a period of 7 h (Working gas phase air, Carbon dioxide absorbent 2 % potassium hydroxide)

Discussion

The principles of a differential respirometer are well known Its features are discussed in textbooks on manometer technique (e g Umbreit et a   ɔ   The use of diethanolamine as carbon dioxide absorbent in a respirometer of this kind has been elucidated by Lukas (1965) whose discussion on respirometric technique is relevant also for the device presented here

In the present article only those circumstances will be discussed that are peculiar for the present apparatus

Leaks are always a problem in respirometry but with careful greasing of the stop cocks and with the entire apparatus under water the linear oxygen consumption shows that this source of error *may be kept negligible Another question is to what* extent the carbon dioxide absorption is efficient It has not been possible to test this directly but since the values for oxygen consumption are linear over a long period of time the carbon dioxide absorption must be judged satisfactory Sensitivity to temperature changes is dependent on the differences in volume between the reaction and the compensation chambers Since this is maximally 4 ml, a temperature change of ± 0.01 °C would mean a difference in the observed reading of oxygen consumption corresponding to ± 0.1 μl The readings are made with an accuracy of ± 0.2 μl, but the variations during the course of an experiment do not permit that the oxygen consumption is calculated with greater accuray than whole microliters per hour and mg dry weight

The obtained values ($QO_2 = 12$) for 15 day old cultured chicken liver are closely corresponding to known values for adult rat liver slices (Krebs and Johnson 1948) Since 15 day old embryonic chicken liver in most respects is equally functionally potent as adult rat liver the obtained values must be judged as quite reasonable It should however be kept in mind as appears from the works of Krebs and Johnson that the respiration rate (oxydation quotient) may vary considerably depending on the composition of the nutritional medium It should also be pointed out that determination of absolute values is of less interest than are the variations under different experimental conditions

A further conclusion that may be drawn from the present results is that the culture method permits satisfactory gas exchange at a level corresponding to the needs of adult mammalian liver, also when the working gas phase in the culture chamber consists of ordinary air

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Reactions within Consecutive Vascular Sections of the Small Intestine of the Cat during Prolonged Hypotension

By

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Received 14 May 1971

Abstract

Haglund, U and O Lundgren *Reactions within consecutive vascular sections of the small intestine of the cat during prolonged hypotension* Acta physiol scand 1972 84 151—163

A plethysmographic technique was utilized to investigate the reactions within consecutive vascular sections of the small intestine of the cat during and after a 2—3 h local arterial hypotension at approximately 55 or 30 mm Hg produced by graded arterial occlusion The sympathetic nerves to the intestine were cut The resistance vessels were dilated during hypotension the dilatation being [illegible]
tension this aut [illegible]
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[illegible] hypotension the whole cardiovascular system seemed to derange progressively to judge by a decline of arterial blood pressure

The vascular bed of the small intestine exhibits like most other vascular circuits autoregulation of blood flow, *i e* flow tends to remain constant despite variations in arterial perfusion pressure This phenomenon has as regards the small intestine been thoroughly investigated by Johnson and coworkers (*cf* Johnson 1960 1964, 1968) Johnson (1964) ascribes autoregulation mainly to an inherent quality of the vascular smooth muscles (the so called Bayliss phenomenon, Bayliss 1902) a conclusion also reached by Lutz (Lutz 1966 Lutz and Henrich 1970) According to this view an increased (decreased) transmural pressure will *per se* cause a vasoconstriction (vasodilatation) since the stretch modulates the inherent smooth muscle activity particularly in the small precapillary vessels Thus it is believed that in most situations local metabolites are less important in the intestinal autoregulation seen upon lowering the perfusion pressure This conclusion seems warranted from the results obtained in the short time experiments reported by Johnson and Lutz How

Fig 1 Schematic illustration of the plethysmographic technique used for studying the reactions within the consecutive vascular sections of the small intestine during local hypotension The technique makes it possible to reduce arterial inflow pressure to the small intestine by means of a clamp around the superior mesenteric artery The intestinal arterial perfusion pressure is recorded from a major branch of the superior mesenteric artery distal to the clamp

ever, during periods of prolonged hypotension, as *e g* during shock, metabolic factors may be of greater importance in determining intestinal peripheral resistance to blood flow

The present study was undertaken to investigate this question and below are reported experiments in which the sympathetically denervated vascular bed of the small intestine was studied during and after prolonged hypotension at two different perfusion pressure levels The investigation was performed utilizing a combination of direct blood flow recording and a plethysmographic technique, originally described for the small intestine by Folkow *et al* (1963, see below) This method makes it possible to follow continuously and simultaneously reactions within resistance vessels, exchange vessels and capacitance vessels as well as to detect changes of mean capillary hydrostatic pressure

Methodological considerations

A Operative procedures and determination of blood flow Experiments were performed on 24 cats weighing 2—4 kg anesthetized intravenously with chloralose (50 65 mg/kg b w after induction with ether The cats had been deprived of food for 12 h and had no obvious signs of intestinal infection

The technique of the present study was described in detail by Folkow *et al* (1963 Below is given a brief description and Fig 1 illustrates schematically the method A segment of the [illegible] ...inder of the [illegible] mesenteric [illegible] ...g it possible [illegible] ...taining the preganglionic sympathetic fibres to the intestinal vessels were cut bilaterally The left adrenal was denervated and the vein from the right one was ligated The vagal fibres to the small intestine were left intact

The cat was heparinized and the blood pressure was recorded from the left femoral artery and from a major branch of the superior mesenteric artery by means of two mercury

TABLE I Values of the capillary filtration coefficient (expressed in ml min × mm Hg × 100 g) calculated from a certain filtration slope at constant arterial (P_a) and venous (P_v) pressures while varying the mean capillary hydrostatic pressure ($\bar{P}_c$) P_v is assumed to be 5 mm Hg

$\bar{P}_c$ mm Hg	P_a mm Hg		
	110	55	35
10	0.047	0.044	0.040
15	0.050	0.050	0.050
20	0.053	0.057	0.067

manometers. The right femoral artery or the left brachial artery were in some experiments cannulated for taking arterial blood samples for pH and P_{CO_2} measurements. Venous outflow from the intestinal segment and its lymph nodes was measured by an optical drop recorder unit. Venous outflow pressure was in most experiments recorded by means of a water manometer coupled to the connection between the superior mesenteric vein and the drop recorder unit.

B Plethysmographic and radioactive isotope techniques To allow a continuous and sensitive recording of changes in tissue volume, the jejunal segment and its lymph nodes were enclosed [illegible]

contents wi[illegible]

In three [illegible]

C Determination of the capillary filtration coefficient It was in most experiments possible to adjust the venous outflow pressure initially so that an "isovolumetric state" was attained. The [illegible]

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To be able to calculate the capillary filtration coefficient (CFC) it is necessary to correlate the rate of outward filtration with the induced change in mean capillary hydrostatic pressure. The extent to which the increase of venous pressure is propagated to the capillary level depends on the relation between pre- and postcapillary resistances of the vascular bed (Pappenheimer 1953). However, all the techniques previously described for determining pre- to postcapillary [illegible] sion see Wallen[illegible] 1963 and in [illegible] 15 mm Hg was [illegible] Folkow *et al* (1963) calculated the error in the CFC determination if the *true hydrostatic* capillary pressure amounted to 5 or 25 mm Hg instead of the assumed 15 mm Hg. It was found that the error thus introduced amounted to only 10—15 per cent as long as the arterial blood pressure is within the normal range around 100 mm Hg.

While thus the error introduced in the calculation of CFC b[illegible] g a certain mean hydrostatic pressure ($\bar{P}_c$), may be negligible when arte[illegible] es [illegible] P_a is "normal" a considerable error can be made in the *estimation when* P_a [illegible] *ustrated in a hypothetical example shown in Table I* where the values of CFC [illegible] ed from one and the same "filtration slope" at constant arterial and venous p[illegible] are compared, assuming different $\bar{P}_c$ values. It is evident from this Table that if the true $\bar{P}_c$ 20 instead of 15 mm Hg the "true" CFC is miscalculated by around 35 per [illegible] a P_a of 35 mm Hg. The changes of CFC observed in this study are however, of a [illegible] greater [illegible] agnitude. Furthermore if P_a and $\bar{P}_c$

remain constant a gradual change in calculated CFC must reflect a true shift in water conductivity of the capillaries

D The acid base balance of the experimental animal In order to maintain a fairly constant arterial pH and P_{CO_2} a bicarbonate solution (10 mekv $NaHCO_3$ per 100 ml 10 % glucose solution) was infused intravenously at a rate of 0 1 ml/min throughout most experiments This procedure was checked in most experiments by determining pH and P_{CO_2} of arterial blood and pH of the intestinal venous blood 3—5 times with an Astrup pH meter (pH meter 27 with a microelectrode unit)

E Experimental procedures After enclosing the intestinal segment in the plethysmograph, the cat was allowed to "rest" for approximately 30 min in order to reach a control steady state in arterial pressure, blood flow and tissue volume Four different types of experiments were then performed

In one series of experiments arterial blood pressure, intestinal blood flow and capillary filtration coefficient were registered for 4—5 h These experiments are referred to as control experiments In a second series of experiments the arterial inflow pressure to the small intestine was reduced in a stepwise fashion by the adjustable clamp The induced changes in blood flow and tissue volume were observed for a few minutes at each pressure level and blood pressure was then again restored to "normal" In a third series of experiments intestinal inflow pressure was lowered to approximately 55 mm Hg for as long as 1 1/2—2 h Changes in intestinal blood flow, intestinal tissue volume and intestinal CFC (see above) were continuously observed In a fourth series of experiments intestinal inflow pressure was lowered to about 30 mm Hg for 2 or 3 h

Results

A Control experiments

In the control experiments in which inflow pressure to the small intestine was left undisturbed, arterial blood pressure tended to decrease approximately 10 per cent during a 4 h period Blood flow resistance remained unchanged or showed a tendency to increase due to a slight decrease of intestinal blood flow CFC was unaltered

B Prolonged hypotension at 55 mm Hg

1 Resistance vessels The upper panel of Fig 2 illustrates the effect of a prolonged local arterial hypotension (55 mm Hg) on intestinal blood flow (squares) and regional flow resistance (triangles) The responses recorded during and after the hypotension period are expressed in per cent of control which is set to 100 Intestinal blood flow then amounted to 26 ± 2.5 ml/min$\times$100 g (mean$\pm$S E , n=23) and resistance, expressed in PRU_{100} (mm Hg$\times$100 g$\times$min/ml, Green 1948) to 4.5 ± 0.45 (n=23) These values are within the 'resting range earlier reported (Lundgren 1967)

Intestinal blood flow and flow resistance were within 5 min reduced to 70—80 per cent of control upon lowering arterial inflow pressure to approximately 55 mm Hg The decrease in flow resistance indicates that a relaxation of the intestinal vascular smooth muscles has occurred A further small decrease of intestinal flow resistance to approximately 65 per cent of control was sometimes observed after 60—80 min of hypotension When the partial arterial occlusion was released the intestinal blood flow returned towards the control level Since, however systemic arterial pressure had been lowered from a mean value of about 110 mm Hg before to around 95 mm Hg after the hypotensive period a decrease in flow resistance was present

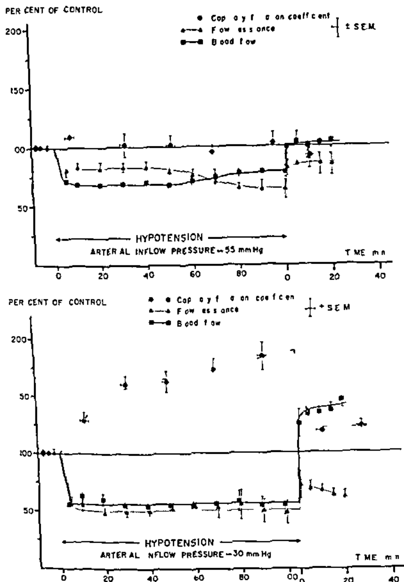


Fig 2 *Upper panel* The effects of a prolonged reduction of arterial inflow pressure to approximately 55 mm Hg on blood flow flow resistance and capillary filtration coefficient (CFC) of the small intestine The results are expressed in per cent of the 'resting' control values Lines drawn by inspection The data were obtained in 5 expts Each CFC value represents a mean value of 10—15 measurements *Lower panel* The effects of a prolonged reduction of arterial inflow pressure to approximately 30 mm Hg on the intestinal vascular bed illustrated as described above The Fig is based on 6 expts Each CFC value presents a mean value of 12 18 determinations

2 *Exchange vessels* No significant change of CFC (Fig 2 upper panel filled circles) occurred during arterial hypotension of 55 mm Hg This suggests that the tone of the precapillary sphincter sections determining the size of the area avail

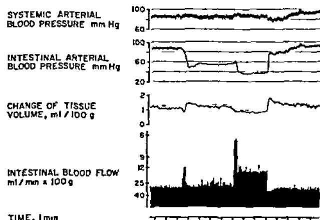


Fig 3 Cat 3.3 kg Th effects of stepwise reductions of arterial per fusion pressure on intestinal blood flow and tissue volume Note that intestinal tissue volume reaches an isovolumetric state (indicated by dotted lines) after each reduc tion in arterial inflow pressure

able for transcapillary water exchange, remained unaltered throughout the hypo tension In absolute terms CFC measured 0 052 ± 0 004 ml/min × 100 × mm Hg (n = 20) during control conditions

3 Mean capillary hydrostatic pressure Fig 3 illustrates that tissue volume remained rather constant upon lowering inflow pressure to 50—55 mm Hg indicating that mean capillary hydrostatic pressure was unchanged (Mellander 1960) Similarly, during the prolonged periods of hypotension at 55 mm Hg no significant change in ean capillary hydrostatic pressure seemed to occur

4 Capacitance vessels No significant change of regional blood volume was observed when arterial inflow pressure was suddenly lowered to 50—55 mm Hg to judge by the insignificant net changes of tissue volume (Fig 3) This is presumably due to the fact that the precapillary autoregulatory adjustments allowed the mean distend ing pressure within the postcapillary vascular section where the capacitance function is mainly located, to remain the same as in the control period

C Prolonged hypotension at 30 mm Hg

1 Resistance vessels The lower panel of Fig 2 illustrates the effect of a prolonged more profound regional hypotension (around 30 mm Hg) on intestinal blood flow (squares) and regional flow resistance (triangles) This figure is based on 6 experi ments in which the local hypotension period amounted to 100 min. The reactions within the consecutive vascular sections are expressed in per cent of control During the control period intestinal blood flow and resistance amounted to 25 ± 2 0 ml min × 100 g (n = 18) and 4 7 ± 0 34 PRU_{100} units (n = 18 respectively

Intestinal blood flow and flow resistance were both rapidly reduced to about 50 per cent of control upon lowering arterial inflow pressure reflecting a marked re laxation of the resistance vessels Both parameters remained at this level throughout hypotension Upon releasing the arterial occlusion intestinal blood flow exceeded the control value, although arterial inflow pressure was now often lower than during the

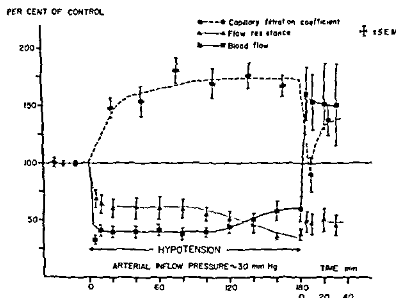


Fig 4 The effects of a prolonged h... blood flow, flow resistance and ca... expressed in per cent of the "rest... were obtained in 6 expts and each (...

initial control period Hence, resistance remained below control and even showed a slight tendency to decrease further with time

In another series of six experiments the arterial inflow pressure was kept at 30 mm Hg for about 3 h (Fig 4) During the control period blood flow and flow resistance amounted to 28 ± 2 0 ml/min × 100 g (n = 19) and 4 3 ± 0 20 PRU_{100}-units (n = 19), respectively Comparing Fig 2 (lower panel) and 4 it is evident that the initial response pattern of the resistance vessels is largely similar in these two series of experiments However, during the additional 80 min of hypotension in Fig 4, intestinal blood flow gradually increased and consequently resistance decreased considerably *Upon releasing the partial arterial occlusion after 3 h of hypotension* blood flow was markedly enhanced as compared to control and only a slight regain of vascular tone was observed

It should be pointed out that after releasing the arterial clamp around the superior mesenteric artery in the experimental series of Fig 2 (lower panel) and 4 the cardiovascular system seemed to derange progressively Thus arterial blood pressure fell at a rate of 20—40 mm Hg each half hour a phenomenon never observed *during* the hypotensive periods

2 Exchange vessels CFC seemed to increase during the first part of the period of profund hypotension, first rather rapidly and then more slowly (Fig 2, lower panel, filled circles) This probably reflects a relaxation of the "precapillary sphincter" sections During the latter half of a 3 h hypotension CFC remained constant

level approximately 75 per cent above control (Fig 4), to decrease somewhat after the hypotensive period though it did usually not return to control

CFC measured 0 019±0 002 (n=21) during the control period of Fig 2 (lower panel) and 0 058±0 003 ml/min×mm Hg×100 g (n=19) during the control period of Fig 4

3 Mean capillary hydrostatic pressure While brief periods of profound hypotension hardly altered mean capillary hydrostatic pressure to judge from the insignificant changes of tissue volume (Fig 3), a slow but steady increase of tissue volume was usually observed during the latter half of a prolonged hypotension Experiments using ^{51}Cr labelled red cells clearly indicated that this volume increase was not caused by any change of regional blood volume Assuming then that this volume augmentation was solely caused by a transcapillary filtration of fluid, it could be calculated that mean hydrostatic capillary pressure was increased 1—2 mm Hg (*cf* Lewis and Mellander 1962) Upon releasing the partial arterial occlusion, tissue volume increased even more rapidly in the absence of any change of regional blood volume The tissue volume augmentation corresponded to a calculated further increase of mean capillary pressure of 2—4 mm Hg However, within 5—15 min after the release, tissue volume tended to level off to reach again an 'isovolumetric state The observed volume increases were not abolished by giving atropine (1 mg/kg b w) to the animal

4 Capacitance vessels A further small decrease of tissue volume probably reflecting reduced blood content was seen upon lowering arterial inflow pressure from 55 to 35 mm Hg (Fig 3)

D The relationship between PRU_{100} and CFC

Fig 5 illustrates the relationship between PRU_{100} and CFC during 4 different experimental conditions *i e* during resting control' (squares), during vasodilatation induced by a close i a infusion of isoprenaline (closed circles) and during prolonged regional hypotension with the inflow pressure kept at approximately 55 (triangles) and 30 mm Hg (open circles) The data obtained during dilatation induced by isoprenaline (isopropylnoradrenaline) were calculated from the results reported by Folkow *et al* (1963) The illustrated results from the hypotensive experiments were all recorded after the first 30 min period of hypotension CFC values obtained during 'resting conditions and during prolonged hypotension at the 55 mm Hg level coincide while during prolonged hypotension at 30 mm Hg larger CFC values were observed for given resistance levels though they never reached the high values obtained when the dilator drug was given

E The relationship between total venous outflow and CFC

Fig 6 illustrates the relationship between intestinal blood flow and CFC during four different experimental conditions *i e* during rest (squares), during neurogenic vasoconstriction (sympathetic stimulation, closed circles) and during prolonged hypo

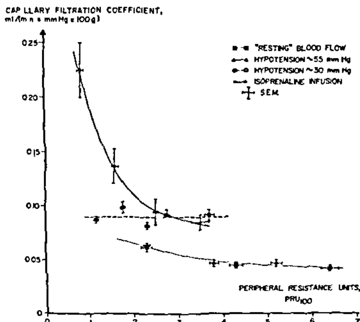


Fig 5 Cumulated data on the correlation between blood flow resistance expressed in PRU_{100}-units and the capillary filtration coefficient of the small intestine during 4 different experimental situations The results during isoprenaline infusion were obtained by Folkow *et al* (1963) Lines were drawn by inspection

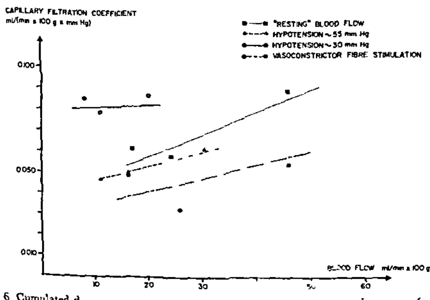


Fig 6 Cumul... coefficient du... fibre stimulat... were calculat... observations

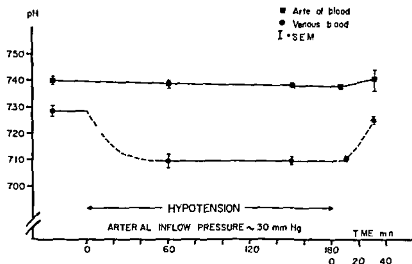


Fig 7 The alterations in pH of arterial blood and of intestinal venous blood before during and after prolonged arterial hypotension at around 30 mm Hg The Fig is based on 6 expts Each point represents a mean value of 3—5 observations

tension at approximately 55 (triangles) and 30 mm Hg (open circles) Regression lines were calculated using the method of least squares The results during sympathetic stimulation (Haglund and Lundgren to be published) were recorded during the steady state phase of neurogenic vasoconstriction (Folkow *et al* 1964) The data from the hypotensive experiments were the same as those in Fig 5 Again the results obtained during resting conditions and during hypotension at 55 mm Hg coincide fairly well while CFC during sympathetic vasoconstriction is lower and during hypotension at 30 mm Hg decidedly higher than during resting conditions at comparable flows

F Blood pH and P_{CO_2} during hypotension

pH and P_{CO_2} of arterial blood and pH of intestinal venous blood were repeatedly determined during most experiments Arterial P_{CO_2} remained constant (mean ± S E 27 ± 0.4 mm Hg n = 15) throughout the experiments A constant pH of the arterial blood was also observed (Fig 7) perhaps mainly due to the slow intravenous infusion of a bicarbonate solution (Methods section D) Intestinal venous pH on the other hand was markedly decreased during hypotension indicating the presence of acid metabolites in the intestinal venous blood and tissue (Fig 7)

Discussion

The present results confirm earlier observations (Johnson 1960 1964 1968) that the sympathetically denervated intestinal vascular bed exhibits a pronounced precapillary autoregulation when the perfusion pressure is lowered (Fig 2 and 4) It seems reasonable, however to assume that the vascular smooth muscle relaxation is predominantly a myogenic phenomenon in the early part of a hypotensive period since

flow resistance fell quite rapidly to reach a new, relatively constant level, while accumulating "tissue metabolites" would have been expected to induce a more gradual reduction During more prolonged hypotension, however, a further, slow decrease of resistance was observed (Fig 4), presumably then caused by accumulating vasodilator "metabolites" This conclusion was further corroborated by the observation that blood flow remained above control after the hypotension (Fig 4)

A lowering of arterial inflow pressure to a skeletal muscle region causes a relatively much more pronounced flow reduction than a corresponding local arterial hypotension in the small intestine (*cf* Lewis and Mellander 1962, Mellander and Lewis 1963), *i e* the autoregulation of blood flow is usually more efficient in the intestine than in skeletal muscle

The magnitude of the capillary filtration coefficient (CFC) is related to the size of the capillary surface area available for fluid exchange and to the number of "pores" per unit capillary surface area (Pappenheimer 1953, Mellander 1960) The size of this area, in turn, is governed by the average activity level of the "precapillary sphincter section" which is usually considered to contribute relatively little to total resistance in most vascular beds Consequently, net changes in total resistance are not closely parallelled by CFC changes, in fact, there are situations when these two parameters change in different directions (see *e g* Folkow *et al* 1964)

In this study of the intestinal vascular bed a lowering of arterial pressure to approximately 55 mm Hg induced a resistance reduction while CFC remained unchanged but when reduced to 30 mm Hg both resistance vessels and precapillary "sphincters" appeared to relax Apparently the precapillary sphincter sections of the intestinal vascular bed are less sensitive than the resistance vessels to a decrease of transmural pressure since the CFC increase is considerably retarded in relation to the prompt resistance decrease Largely the opposite seems to be true for the muscle vessels (Cobbold *et al* 1963) On the other hand the "sphincter sections" of the intestine appear to respond more to dilator "metabolites", as judged by the more gradual increase of CFC observed during the hypotension at 30 mm Hg in the face of a largely unaltered blood flow A vasodilator drug like isoprenaline on the other hand causes a concomitant and largely parallel relaxation of both resistance and "sphincter" sections (Fig 5) while neurogenic vasoconstriction decreases CFC decidedly more than blood flow (Folkow *et al* 1964) These observations again suggest that the precapillary sphincter section of the intestinal vascular bed is much more sensitive to local chemical factors than the resistance vessels in contrast to the situation in skeletal muscle vascular bed Furthermore in the latter vascular bed CFC remains unaltered in the steady state phase of vasoconstriction or may even increase above control (Cobbold *et al* 1963) presumably a result of metabolic accumulation relaxing the precapillary sphincters despite the neurogenic influence again in contrast to the intestinal vascular bed

The possibility exists that the rise in intestinal CFC observed during hypotension might at least partly reflect an increase of capillary "porosity" *e g* by an accumulation of substances affecting permeability or by a hypoxic damage to the endothe-

cells It is however, difficult to imagine that such mechanisms could be able to affect capillary permeability already in the initial stages of hypotension when in fact, the rate of increase of intestinal CFC seemed to be most pronounced Moreover the capillary endothelium appears to be quite resistant to relative ischemia and hypoxia (Korner 1959)

It was proposed by Folkow *et al* (1963) that the intestinal autoregulatory response mainly localized to the precapillary vessels may be primarily designed to keep mean capillary hydrostatic pressure rather than blood flow constant The results of the present study strongly favour this view since it was repeatedly demonstrated that mean capillary hydrostatic pressure remained surprisingly constant even when arterial blood pressure and intestinal blood flow, were markedly reduced (Fig 3) This sensitive mechanism must be of great importance in preventing an excessive net fluid loss from the blood vessels across the large and highly permeable capillary surface area of the small intestine (Folkow *et al* 1963 Johnson 1964)

It was observed that intestinal volume increased slowly during the latter part of a 2—3 h hypotension at 30 mm Hg an increase that was accentuated when the partial arterial occlusion was released By means of [51]Cr labelled red cells it could be shown that the regional blood volume did not increase It is not possible with the present techniques to differentiate between transcapillary filtration and secretion It seems however less plausible that secretion an energy consuming process should be in creased when blood flow was most markedly reduced *i e* at an arterial inflow pres sure of 30 mm Hg Furthermore it was not possible to abolish the tissue volume increases by the administration of atropine

Assuming then that the tissue volume augmentations were solely due to a trans capillary filtration secondary to an increase of capillary pressure it could be calcu lated that this pressure increased 1—2 mm Hg during the latter half of a 30 mm Hg hypotension increasing another 2—5 mm Hg above control upon release of the partial occlusion Thus the autoregulatory mechanism normally keeping intestinal capillary pressure remarkably constant seems to derange during prolonged severe hypotension probably a result of accumulating tissue metabolites causing a de crease of the pre to postcapillary resistance ratio beyond that induced by the normal autoregulatory mechanism

Arterial blood pH and P_{CO_2} were kept within the normal range (in the cat 7 38 ± *0 06 and 28 ± 4 mm Hg respectively mean ± S.D Fink and Schalm 1963*) by an i v bicarbonate infusion and arterial pH remained virtually unchanged even after lowering intestinal arterial pressure to 30 mm Hg for three hours (Fig 7) pH of intestinal venous blood was markedly lowered during the hypotensive period but returned towards control upon pressure restoration (Fig 7) Despite the normaliza tion of blood pH and P_{CO_2} after the prolonged hypotensive periods at 30 mm Hg cardiovascular *function* deranged progressively and the intestinal resistance vessels never regained their tone after such a long period of severe regional hypotension (Fig 4) When the skeletal muscles of the cat s hindquarters in a similar way were exposed to prolonged severe hypotension no such cardiovascular derangement

seemed to occur after the hypotensive period and regional vascular tone seemed to be rapidly restored (Lewis and Mellander 1963) It thus appears that some 'factor(s)' is released from the intestine during more profound restrictions of its blood supply, a 'factor' which has deleterious effect not only on the regional vessels but also on the cardiovascular system as a whole Such an 'intestinal factor' has earlier been discussed in connection with irreversible hemorrhagic shock (see *e g* Lillehei 1957)

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General Characteristics of Sympathetic Activity in Human Skin Nerves

By

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Abstract

Hagbarth, K.-E., R. G. Hallin, A. Hongell, H. E. Torebjörk and B. G. Wallin. *General characteristics of sympathetic activity in human skin nerves.* Acta physiol. scand. 1972. *84.* 164—176.

Synchronized bursts of efferent sympathetic impulses, appearing either spontaneously or triggered by various peripheral stimuli, were recorded with microelectrodes inserted percutaneously into skin nerve fascicles in alert, adult subjects. The signals were abolished by sympathetic ganglion [illegible] Many *of the sy*[illegible] vasocons[illegible] activity [illegible] occurred largely independently of spontaneous blood pressure variations, indicating a relative lack of baroreflex control of the vasoconstrictor outflow to the skin. A loose coupling was observed, however, between the resting respiratory rhythm and the spontaneous sympathetic bursts in the skin nerves.

Sympathetic activity in human muscle nerves, appearing as spontaneous bursts of impulses grouped in the pulse rhythm, was first described by Hagbarth and Vallbo (1968). Their findings were recently confirmed by Delius *et al.* (1971 a), who noted in addition an inverse relationship between the strength of the pulsative sympathetic outflow in the muscle nerves and spontaneous blood pressure fluctuations. According to Hagbarth and Vallbo a similar type of activity is not to be found in skin nerve fascicles. During an extended search for sympathetic signals in such fascicles, however, we now and then observed spontaneously occurring bursts of impulses which appeared in seemingly random fashion without any obvious relation to pulse rhythms or spontaneous blood pressure fluctuations. A systematic study of these neural phenomena in human skin nerve fascicles has now been undertaken. The results to be presented lead to the conclusion that the signals derive from groups of postganglionic sympathetic fibres which in awake relaxed subjects exhibit a fluctuating resting discharge of variable strength and which are easily activated by sudden arousal stimuli. All of the triggered and many of the spontaneously occurring sympa-

thetic impulse volleys were succeeded by phasic changes in skin resistance and/or plethysmographic signs of vasoconstriction within the innervation zone of the skin fascicle impaled As will be shown in a succeeding report (Delius *et al* 1971 c) the fluctuating discharge can be elevated or suppressed in various ways, *e g.* by changing the room temperature or by altering the attentive or emotional state of the subject

Methods

Subjects The investigations were made on 13 subjects, 9 men and 4 women, ranging in age from 24—49 years Four of them served as an experimental subject 2—4 times None of them showed signs or symptoms of any neurological or cardiovascular disease All had been instructed beforehand about the experimental procedures They remained calm and relaxed during the recordings and afterwards stated that they had experienced no severe discomfort or pain

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Technical details concerning recordings of nerve activity, blood pressure, ECG and respiratory rate have been described previously (Delius *et al* 1971 a)

Changes in skin resistance were recorded by two Ag AgCl electrodes in a Wheatstone bridge arrangement One electrode was taped to the glabrous skin within the receptive field of an impaled median nerve fascicle and the other was placed some distance outside this area, usually on the volar side of the wrist

Pulse plethysmography was recorded by means of an air filled plastic cylinder applied around the distal phalanx of a finger and attached to a crystal pressure transducer (EMT 510, Elema-Schönander Ltd, Stockholm, Sweden)

Results

Pattern of spontaneous efferent activity in skin nerves

Fig 1 shows an example of the fluctuating neural activity that can be recorded from certain regions within skin nerve fascicles in awake, relaxed subjects Like the multiunit sympathetic bursts in muscle nerves the spontaneous skin nerve activity under consideration had a main frequency content in the range of 700—1600 Hz which caused a characteristic sound in the loudspeaker differing from that evoked by afferent mechanoreceptor discharges Unlike the efferent pulse synchronous bursts in muscle nerves, however, the multiunit impulse barrages in the skin nerves occurred in a more random fashion, they sometimes lasted for 2 s or more and they were sometimes repeated at frequencies within the range of 10—30/min The activity

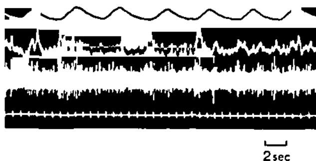


Fig 1 Spontaneous efferent multi unit nerve activity recorded in a median nerve fascicle innervating the palm of the hand of an awake relaxed subject *Upper tracing* shows respiratory move ments (inspiration upwards) *Second tracing* is a mean voltage neurogram obtained from the original nerve record (*third tracing*) by an electronic integra tion technique (time constant 0 11 s), *Fourth tracing* shows ECG

similar appearance in skin nerves of the upper and lower extremities and so far no significant difference has been found between fascicles innervating glabrous and hairy skin As a rule no coupling to heart rate was detected although exceptionally a few bursts appeared in the integrated neurogram with an interval corresponding to the pulse frequency (see Fig 4 A left) It was then more common to find some correla tion between the spontaneous bursts of impulses and the respiratory rhythm, especi ally during deep respiration Also as will be shown below, bursts of impulses could be triggered by sudden arousal stimuli

Evidence for the sympathetic origin of the signals

The outcome of the following tests showed in a convincing way that the signals de scribed were of sympathetic origin

a) *Effect of nerve blocks with local anaesthetics* When 1 % Lidocaine was injected around the nerve distal to the recording site the spontaneous bursts remained as be fore whereas afferent signals no longer could be elicited by mechanical skin stimuli within the receptive field However when the local anaesthetic was injected proximal to the recording site the spontaneous activity promptly disappeared proving the efferent origin of the impulses

b) *Effector responses* A correlation was found between bursts of nerve activity and changes in pulse plethysmograms and skin resistance curves suggesting a causal rela tionship with vasomotor as well as sweat gland activity (see below) Also, as will be shown in a succeeding report manoeuvres known to alter vasomotor tone in the skin were accompanied by corresponding alterations in the strength of the neural impulse barrages (Delius *et al* 1971 c)

c) *Effect of blocking sympathetic ganglia* If the spontaneous activity is of sympa thetic origin it should be blocked by a ganglion blocking agent Fig 2 shows the out come of such an experiment where trimethaphan was given by slow intravenous infusion in a 35 year-old man while recording from a peroneal skin nerve fascicle In this case the initial spontaneous activity was very inconspicuous and it soon dis

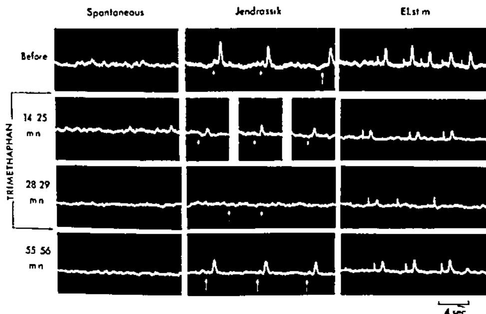


Fig 2 The effect of intravenous infusion of a sympathetic ganglion blocking drug (tri methaphan) on ...
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appeared after the start of the infusion However, distinct reflexly induced bursts of impulses could easily be triggered by painful electrical shocks or sudden clenching of the contralateral hand Such triggered volleys were still seen after the spontaneous activity had ceased but as shown in the figure the response gradually declined and finally disappeared during a prolonged infusion After the infusion of trimethaphan was stopped the responses reappeared During the whole experiment afferent nerve volleys were now and then elicited by touch stimuli within the receptive field and, as expected, these responses were not influenced by the drug

Similar results were obtained when recording from skin nerve fascicles in two patients with essential hypertension In these cases the spontaneous resting activity was much more pronounced than in the experiment illustrated in Fig 2 Both the spontaneous and the induced activity disappeared completely in one case and almost completely in the other during the infusion of trimethaphan

Relative strength and abundance of sympathetic signals in various skin nerves and various individuals

The finding that the sympathetic signals can be recorded only from certain intra fascicular regions agrees with histological observations that unmyelinated fibres are grouped in certain regions of the nerve bundles (Gasser 1955) No systematic attempts

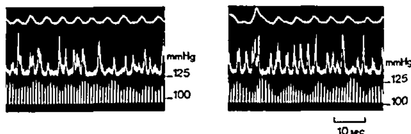


Fig 3 Simultaneous recordings of intraarterial blood pressure (*lower tracings*) and sympathetic skin nerve activity (*middle tracings*) under resting conditions Left and right recordings are two samples from the same experiment Nerve records from peroneal nerve fascicle innervating hairy skin (only integrated neurograms included) *Upper tracings* show the respiratory movements (inspiration upwards)

were made to map the intrafascicular spatial distribution or compare the absolute strength of the integrated sympathetic activity as recorded from different skin nerves of different individuals Nevertheless certain interindividual differences were noted with respect to the relative predominance of the spontaneous sympathetic activity As when searching for sympathetic activity in muscle nerves we found the strongest sympathetic skin nerve discharges in those 4 subjects who were over 40 years of age and most of the figures presented were obtained from these persons

No obvious difference was noted in the strength and abundance of the sympathetic signals in nerves innervating glabrous and non glabrous skin or in nerves of the upper and lower extremities

Relation between sympathetic outflow and spontaneous blood pressure fluctuations

In 4 of the subjects the i a blood pressure was recorded for 1—2 h in parallel with sympathetic skin nerve activity Under resting conditions no correlation was detected between the sympathetic bursts and the spontaneous variations in arterial blood pressure Fig 3 (left) shows an example of rapid rather small fluctuations of systolic blood pressure and Fig 3 (right) demonstrates variations which are slower and of somewhat higher amplitude In both cases the sympathetic bursts appeared randomly without any apparent relation to the blood pressure changes The correlation (as judged by visual inspection) was equally poor also when comparison was made with diastolic or mean pressure fluctuations

Relation between sympathetic outflow and respiratory rhythm

The sympathetic skin nerve volleys recorded under resting conditions were sometimes coupled to the respiratory rhythm with the bursts appearing more often during the inspiratory than during the expiratory phase (Fig 4 A During normal quiet breathing the coupling was loose and variable and the bursts usually continued uninterrupted during apnoeic periods (Fig 4 B) However a spontaneous sigh or a single voluntary deep inspiration (Fig 5 A) regularly evoked a pronounced burst of neural

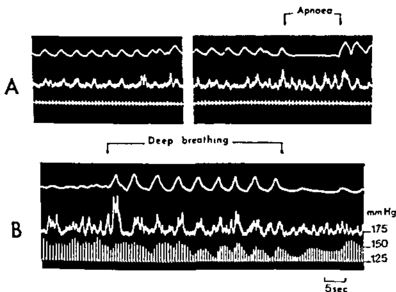


Fig 4 Examples of the relation between sympathetic skin nerve activity and the respiratory rhythm during normal respiration (*A, left*), voluntary apnoea (*A, right*) and voluntary deep respiration (*B*) *Upper tracings* Respiratory movements (inspiration upwards) *Middle tracings* Integrated sympathetic skin nerve activity recorded in the median (*A*) or peroneal nerve (*B*) *Lower tracings* ECG (A) or intraarterial blood pressure (B)

activity with a latency of about 0 4—0 5 s (when recording from the median nerve at the elbow) Rhythmic deep respiration or voluntary hyperventilation usually caused a grouping of the bursts in the respiratory rhythm as Fig 4 B In this figure the systolic blood pressure also shows rhythmic variations with a frequency which after a while approximates that of the respiration It can be seen that (in contrast to the finding in muscle nerves) the sympathetic skin nerve activity correlates much better to the respiratory movements than to the systolic blood pressure variations

Stimuli triggering bursts of sympathetic activity

In most subjects bursts of sympathetic skin nerve activity could be triggered by a variety of stimuli A single rapid breath or a sudden chest compression was very potent in this respect and when recording from the median nerve at the elbow, the reflex response appeared with a latency of about 0 5 s (Fig 5 A and B) Usually, the triggered sympathetic bursts were stronger than the spontaneously occurring ones The same type of response was evoked by electrical shocks applied anywhere on the body surface (Fig 5 C) Most of the triggered sympathetic bursts had a duration of about 1 s but some of those elicited by electrical shocks lasted for 1 5 s or more Fig 5 D illustrates such a long duration response which in addition had two distinct peaks

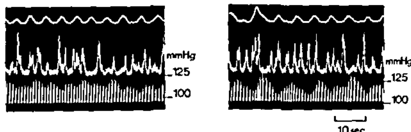


Fig 3 Simultaneous recordings of intraarterial blood pressure (*lower tracings*) and sympathetic skin nerve activity (*middle tracings*) under resting conditions Left and right recordings are two samples from the same experiment Nerve records from peroneal nerve fascicle innervating hairy skin (only 'integrated neurograms included) *Upper tracings* show the respiratory movements (inspiration upwards)

were made to map the intrafascicular spatial distribution or compare the absolute strength of the integrated sympathetic activity as recorded from different skin nerves of different individuals Nevertheless, certain interindividual differences were noted with respect to the relative predominance of the spontaneous sympathetic activity As when searching for sympathetic activity in muscle nerves, we found the strongest sympathetic skin nerve discharges in those 4 subjects who were over 40 years of age and most of the figures presented were obtained from these persons

No obvious difference was noted in the strength and abundance of the sympathetic signals in nerves innervating glabrous and non glabrous skin or in nerves of the upper and lower extremities

Relation between sympathetic outflow and spontaneous blood pressure fluctuations

In 4 of the subjects the i a blood pressure was recorded for 1—2 h in parallel with sympathetic skin nerve activity Under resting conditions no correlation was detected between the sympathetic bursts and the spontaneous variations in arterial blood pressure Fig 3 (left) shows an example of rapid rather small fluctuations of systolic blood pressure and Fig 3 (right) demonstrates variations which are slower and of somewhat higher amplitude In both cases the sympathetic bursts appeared randomly without any apparent relation to the blood pressure changes The correlation (as judged by visual inspection) was equally poor also when comparison was made with diastolic or mean pressure fluctuations

Relation between sympathetic outflow and respiratory rhythm

The sympathetic skin nerve volleys recorded under resting conditions were sometimes coupled to the respiratory rhythm with the bursts appearing more often during the inspiratory than during the expiratory phase (Fig 4 A During normal quiet breathing the coupling was loose and variable and the bursts usually continued uninterrupted during apnoeic periods (Fig 4 B) However a spontaneous sigh or a single voluntary deep inspiration (Fig 5 A) regularly evoked a pronounced burst of neural

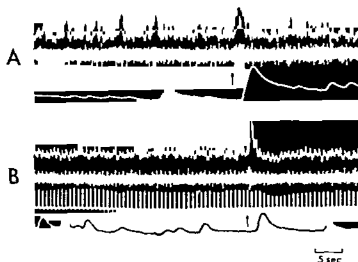


Fig 6 Relationship between sympathetic skin nerve activity recorded in the median nerve at the wrist and corresponding effector organ responses
A shows correlation to skin resistance changes (*lower tracing*) recorded from a skin area within the innervation zone of the fascicle impaled Decrease of skin resistance shown as upward deflection *Upper and second tracings* of A and B show mean voltage and original neurograms, respectively Arrow indicates application of sudden painful stimulus (skin pinching)
B sho … n resistance (*lower tracing*) and pulse
p … iervation zone The arrow indicates a
si … a transient reduction in skin resistance
a

in a monotonous way In general the more unexpected and novel the stimulus the more intense was the response In the sequence of deep breaths illustrated in Fig 4 B it is obvious that the strongest sympathetic burst is evoked by the first inspiration, and similar 'novelty' effects were seen with most types of stimuli mentioned It also happened when a series of identical stimuli were applied that individual responses fell out of a sequence and if the frequency of stimulation was high *e g* electrical shocks applied at 1/s or more, most of the stimuli failed to evoke a reflex response

One of the subjects showed a striking example of how diversion of attention could influence the outcome of the tests During a series of repeated taps against the skin of the leg (outside the fascicular receptive field) the regularly occurring sympathetic reflex responses in the peroneal nerve temporarily disappeared as soon as the attention of the subject was focused on his left arm where an adjustment of the intra arterial catheter was made

Relation between sympathetic skin nerve activity and effector organ responses

Recordings of skin resistance and/or pulse plethysmography were sometimes made in parallel with the nerve recordings and Fig 6 shows representative exemples of the results

Skin resistance A fairly good correlation was observed between sympathetic skin nerve discharges and changes in skin resistance measured in the receptive field of the fascicles impaled This was most evident for the reflex responses triggered by external arousal stimuli Such neural bursts were always followed by reductions in skin resistance (often quite large) with a latency of 1—1 5 s and sometimes lasting more than 5 s Fig 6 A shows an example of such an evoked response However for the spontaneous resting activity the correlation was less pronounced Not all spontaneous sympathetic bursts were followed by changes in skin resistance and for those that were the relation between the intensity of the bursts and the magnitude of the skin resistance reductions varied considerably (Fig 6 A) Also spontaneous shifts in skin resistance occurred which were not preceded by visible changes in neural outflow (Fig 6 B)

Pulse plethysmography A few times pulse plethysmographic recordings were made from a finger, supplied by the skin fascicle impaled In these cases most of the spontaneously occurring sympathetic bursts were not followed by any visible change in the plethysmogram However, sympathetic reflex discharges triggered by external stimuli were usually followed by changes in the pulse plethysmogram (Fig 6 B) Like the skin resistance changes the vasomotor responses were sluggish having a latency of 1—1 5 s and a duration of 5—10 s

Discussion

Search for and identification of sympathetic signals The tests to ascertain that the signals were of sympathetic origin did not have to be repeated in each experiment as the multiunit signals described appeared in such a characteristic way and had such a frequency content that in the loudspeaker monitoring they always were easily recognized and discriminated from signals in myelinated cutaneous afferents A constant barrage of signals in large mechanoreceptive afferents was often seen in recordings from fascicles supplying hairy skin (*cf* Vallbo and Hagbarth 1968) but it was usually possible to find *intrafascicular recording sites where the sympathetic* signals stood out clearly against this afferent steady state discharge A certain constant admixture of non sympathetic signals in the integrated neurograms was considered to be of minor importance since no major attention was paid to the absolute *magnitude of the integrator output it was only changes of integrated activity that* were taken into consideration

As judged by the minute electrode movements required to bring sympathetic multiunit signals in or out of focus the signals derived from groups of active sympathetic fibres lying close to the recording surface One may predict however that as a greater number of sympathetic fibre groups are recruited with increasing strength of the outflow there will be an increasing probability that sympathetic signals will appear within the recording range of an electrode placed randomly within a fascicle *This may explain why large efferent volleys* like those triggered by electrical shocks usually had a wider spatial distribution than smaller spontaneous bursts It

may also explain why in some subjects clearly discernible sympathetic signals could be recorded from a few scattered regions within the fascicles, whereas in other subjects the discharges were stronger and more widely spread However, more systematic and quantified evidence is needed to determine whether there are any constant interindividual differences in strength of the sympathetic outflow and to check whether it was a coincidence in the present study that the strongest spontaneous sympathetic discharges were seen in subjects over 40 years of age

Vasoconstrictor and sudomotor signals So far, the recordings from selected sites in different human skin nerves have given no evidence of topographically separated sympathetic fibre groups which differ from each other with respect to firing patterns or response characteristics On the contrary, sympathetic fibre groups in different skin nerves exhibited similar response characteristics and similar fluctuations in their spontaneous discharges, which were quite different from the pulsative sympathetic discharges seen in muscle nerves However, no simultaneous recordings have been made as yet from different skin nerves and therefore it is unknown whether randomly occurring spontaneous fluctuations in the outflow appear in parallel in sympathetic fibres supplying different skin areas

After a delay of 1—1 5 s most efferent nerve volleys evoked in fascicles supplying glabrous skin were followed both by changes in skin resistance and plethysmographic signs of vasoconstriction within the fascicular innervation zone In addition, skin resistance changes with similar latency were seen after many of the spontaneously occurring neural bursts This indicates that the sympathetic signals originate in part from sudomotor fibres but that vasoconstrictor signals probably also are involved in the discharges Previous studies have shown that both changes in skin resistance and plethysmographic signs of finger vasoconstriction tend to occur in response to deep inspirations, auditory stimuli and electrical shocks but there is also evidence that the postganglionic pathways for the two responses are mutually independent (Prout 1967, Furedy and Gagnon 1969) Single — rather than multiunit — recordings are probably required to distinguish between sudomotor and vasoconstrictor impulses That such recordings are possible in man was recently shown by Hallin and Torebjork (1970), who in the median nerve succeeded in isolating a single sympathetic unit which they identified as being sudomotor On the other hand signals in vasoconstrictor fibres are probably responsible for the fact that by cooling the subject one can cause an enhancement of the overall sympathetic activity in skin nerves (Delius *et al* 1971 c)

It is still questionable whether vasodilator fibres are present in human skin nerves (Fox and Edholm 1963) and consequently there is no strong reason to believe that such fibres contribute to the neural discharges recorded Piloerector impulses might possibly be involved in efferent discharges recorded from fascicles supplying hairy skin but we have no positive evidence supporting this suggestion since spontaneous and evoked sympathetic discharges were quite similar in fascicles supplying hairy and glabrous skin

Comparison of sympathetic activity in skin and muscle nerves

a) *Resting conditions.* As previously shown (Delius *et al.* 1971 a), the sympathetic activity recorded from human muscle nerves is modulated by the pulse rhythm and also by spontaneously occurring slow blood pressure waves, indicating an efficient dynamic and static baroreflex control of the sympathetic outflow to the vascular bed of skeletal muscles. The present study shows that quite different neural mechanisms are involved in the control of the sympathetic outflow to the skin. In fact the characters of the spontaneous patterns of sympathetic activity in muscle and skin nerves are so different that the two patterns can be used as a fairly safe means of differentiation between 'pure' skin and muscle nerve fascicles. On those occasions when the electrode, as judged by afferent test stimuli, had impaled a mixed nerve fascicle we occasionally saw a mixed type of sympathetic activity, but, as mentioned above, fascicles of this type were avoided in the present study.

Particular attention was paid to the question of whether any signs of pulse rhythmicity or correlation to spontaneous blood pressure waves could be detected in the sympathetic skin nerve activity. On the whole we found no such correlations indicating dynamic and/or static baroreflex control. However, these results can apparently not be applied to all mammalian skin nerves since Walther, Iriki and Simon (1970) regularly found pulse synchronous sympathetic discharges in nerves supplying the skin of the rabbit's ear. It may also be argued that in our recordings a baroreflex control of the vasoconstrictor outflow to the skin was obscured by the presence of sudomotor impulses not subjected to such control. In this connection it should be noted, however, that no significant change towards a more pulse synchronous pattern was seen during cooling manoeuvres when presumably the enhanced sympathetic outflow is purely vasomotor (Delius *et al.* 1971 c). Also blood flow measurements in cats have shown that blood pressure changes affect the blood flow in skin much less than in skeletal muscles (Löfving 1961).

b) *Reflex responses.* It was reported previously that sudden inspirations, chest compressions or painful electrical shocks against the skin caused a temporary inhibition of the sympathetic bursts recorded from muscle nerves (Delius *et al.* 1971 a). In contrast, the present investigation has shown the same stimuli to have an excitatory effect on sympathetic neurons destined to the skin of the extremities. In animal experiments similar antagonistic reactions in two sympathetic nerves were only recently found (Walther *et al.* 1970, Iriki *et al.* 1971). However, they have long been predicted from the results of regional blood flow measurements (for review see Appenzeller 1970), and as shown in other studies many manoeuvres have different effects upon the sympathetic outflow in human skin and muscle nerves (Delius *et al.* 1971 b and c).

Animal experiments have shown that sympathetic reflex responses triggered by electrical stimulation of various peripheral nerves can be mediated both via spinal and supraspinal pathways (*e.g.* Coote and Downman 1966, Sato *et al.* 1969). In man Gilliatt, Guttman and Whitteridge (1948) found that the vasoconstriction in

the hand in response to a deep breath is a spinal reflex In the present investigation when recording from the median nerve at the elbow, the sympathetic responses to an electrical shock and a deep breath both had a latency of about 0 4—0 5 s which probably is short enough for a spinal reflex However, the long duration of some of the sympathetic reflex bursts is compatible with activity in more than one reflex arc and for the double peak response shown in Fig 5 D this seems a very likely possibility Consequently although a more systematic study of reflex latencies is required before valid conclusions can be drawn the data obtained so far agree with the notion that both spinal and supraspinal reflex arcs are involved

Genesis of sympathetic rhythms in skin nerves

Spontaneous, more or less rhythmic fluctuations are known to occur both in the skin resistance and in the vasomotor tone of the fingers (Hensel and Bender 1956, Figar 1965), and some of these fluctuations are undoubtedly due to variations in the strength of the sympathetic outflow However the effector organs respond after a relatively long delay and in a sluggish way to the sympathetic impulse volleys (*cf* Fig 6) and thus the responses will tend to fuse when there are short intervals between the neural bursts Besides being sluggish the effector organs may be subjected also to hormonal or local metabolic influences altering their responsiveness to changes in sympathetic outflow

Many of the spontaneous sympathetic impulse volleys seen in the skin nerves were probably induced by uncontrolled random external stimuli or sudden changes in the attentive state of the subject (*cf* Delius *et al* 1971 c) However such randomly occurring orienting reactions' (Figar 1965) can hardly explain the rhythmical appearance of the sympathetic bursts The coupling to the respiratory rhythm during normal quiet breathing was loose and irregular and the sympathetic rhythms persisting during periods of apnoea apparently occurred independently of rhythmic feed back from intrathoracic or abdominal receptors The present results agree with the notion that there are certain inherent rhythms prevailing in the central sympathetic structures governing the vasoconstrictor and sudomotor outflow to skin rhythms which can be modified by a variety of influences (*cf* Koepchen 1962 Weidinger und Leschhorn 1964)

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Manoeuvres Affecting Sympathetic Outflow in Human Skin Nerves

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Abstract

Delius, W, K-E Hagbarth, A Hongell and B G Wallin *Manoeuvres affecting sympathetic outflow in human skin nerves* Acta physiol scand 1972 *84* 177—186

Sympathetic activity was recorded from human skin nerves during the execution of a variety of different manoeuvres The neural activity (composed of vasoconstrictor and sudomotor impulses) was regularly affected by emotional, thermal and respiratory stimuli Mental stress, body cooling and increased respiratory movements all increased the sympathetic outflow, whereas the activity decreased during moderate body warming and when the subject was calm and relaxed Manoeuvres known to cause changes in baroreceptor activity usually did not affect the sympathetic outflow to the skin When comparing neural and effector organ responses during the different manoeuvres the changes in sympathetic activity correlated well with changes in skin vascular resistance (either measured simultaneously or as reported in the literature) A comparison of the sympathetic responses recorded from human skin and muscle nerves was also made It was concluded that most manoeuvres had different effects in the two nerve types, thereby confirming earlier indirect evidence for selective regional control of the sympathetic outflow

Spontaneous sympathetic activity has different characteristics in human muscle and skin nerves The activity recorded from muscle nerves consists of bursts of impulses occurring pulse synchronously in short irregular sequences, separated by periods of relative silence The impulses cause vasoconstriction and their occurrence is intimately related to spontaneous blood pressure variations suggesting an inhibitory baroreflex influence on the sympathetic outflow (Delius *et al* 1971 a) The sympathetic activity recorded from skin nerves, on the other hand, consists of a mixture of vasoconstrictor and sudomotor impulses which under resting conditions appear in irregular bursts of varying duration showing neither pulse synchronous grouping nor correlation to blood pressure fluctuations (Hagbarth *et al* 1971)

Apart from these differences in the character of the resting sympathetic outflow, regional blood flow measurements have also indicated functional differences between the nervous control of the vascular beds innervated by the two nerve types A preceding paper described sympathetic muscle nerve activity during various manoeuvres causing circulatory adjustments (Delius *et al* 1971 b) In order to extend the com-

parison between the different types of sympathetic outflow the same manoeuvres were also performed while recording sympathetic skin nerve activity These results are described in the present report The responses differed considerably in the two nerve types, confirming earlier indirect evidence for selective regional control of sympathetic outflow (Mellander and Johansson 1968)

Methods

was applied on the wrist and inflated to 60 mm Hg when blood flow measurements were performed

Vascular resistance in 100 ml tissue (skin muscle) was calculated as mean arterial blood pressure in mm Hg divided by flow in ml 100 ml [1] min [1]

General procedure and manoeuvres used Except for the fact that skin nerve fascicles were

Results

Mental strain and emotional stimuli

During recordings of sympathetic skin nerve activity it was obvious that the intensity of the sympathetic activity often was related to the emotional state of the subject In general the activity increased during periods of mental stress and decreased when the subject was relaxed and comfortable This was most evident during short periods of fairly well defined mental strain as when the subject was suddenly asked to perform mental arithmetic Such tests were made 11 times in 5 subjects and Fig 1 shows a representative example Both the sympathetic nerve activity and blood pressure show pronounced increases with the change in nerve activity preceding the blood pressure rise In a few experiments where simultaneous hand blood flow measurements were made it was seen that the increased neural activity could be accompanied either by vasoconstrictor or vasodilator responses As expected the increase in sympathetic activity during mental stress was followed by transient reductions in galvanic skin resistance

Similar nervous responses although less intense were also elicited by pleasant (the subject being offered a cigarette or a cup of coffee and other less well defined stimuli (the subject talking somebody entering the room In fact almost any type of stimulus or manoeuvre could when first performed be associated with an initial phase of increased sympathetic skin nerve activity but this novelty effect usually was reduced or disappeared after a few repetitions

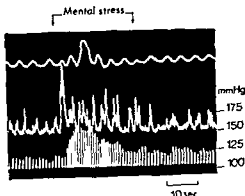
The neural activity often increased as soon as a subject was informed of a forthcoming manoeuvre and an example of such a preparatory response is seen in Fig 6

Fig 1 The effect of suddenly asking the subject to solve an arithmetical problem on sympathetic activity recorded in a peroneal nerve fascicle innervating hairy skin

Upper tracing Respiratory movements (inspiration upwards)

Middle tracing 'Integrated' sympathetic nerve activity obtained from the original nerve recording (not shown) by an electronic integrating circuit (time constant 0 11 s)

Lower tracing Intra arterial blood pressure Note that the increase in sympathetic activity precedes the blood pressure rise



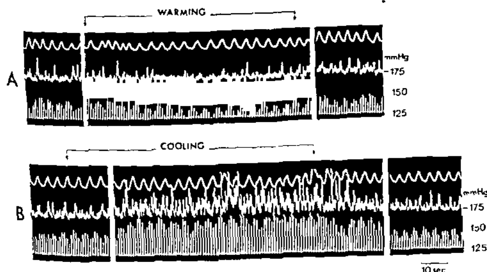


Fig 2 The effect of body warming and cooling on sympathetic activity recorded in a median nerve fascicle innervating glabrous skin Same tracings as in Fig 1 The breaks in the recordings in A comprise approximately 30 s each whereas the first break in B comprises 2 5 min and the second 1 min

This was particularly evident in some subjects who had experienced an unpleasant stimulus several times, where the mere suggestion of the test being repeated again often resulted in a marked increase of the sympathetic skin nerve activity In subjects who were distressed by electrical shocks against the skin this type of conditional response could be elicited merely by approaching the stimulating electrodes towards the skin In most cases when skin nerve activity increased as a result of mental stress the reaction was short lasting and receded completely within 20—30 s after the end of the stimulus Occasionally, however short periods of emotional stimuli could elicit an increase of sympathetic outflow lasting several minutes Also, during the

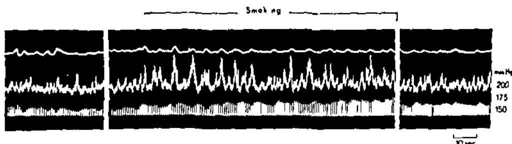


Fig 3 The effect of smoking a cigarette on sympathetic activity recorded in a median nerve fascicle innervating glabrous skin Same tracings as in Fig 1 and 2 The first break in the recording comprises approximately 20 s and the second 2 5 min

course of long recording sessions seemingly spontaneous slow changes in the intensity of the sympathetic skin nerve activity took place, so that the general activity level could be higher or lower at the end of the experiment than at the beginning

Thermal stimuli
The intensity of the sympathetic skin nerve activity was also dependent upon room temperature As shown in Fig 2, warming the subject reduced the sympathetic activity whereas body cooling markedly increased the intensity of the bursts These nervous reactions were accompanied by parallel slow changes in blood pressure (both systolic and diastolic) The findings were similar in all seven subjects in whom the test was made but the responses were not always equally pronounced There seemed to be some correlation between the magnitude of the responses and the subject's own experience of the stimulus *i e* the cooler the subject felt the more did his sympathetic skin nerve activity increase During cooling the hand blood flow decreased and the normal spontaneous skin resistance variations were reduced or even totally abolished

Smoking
The effect of smoking a cigarette was tested twice while recording sympathetic skin nerve activity In both cases the *intensity of the sympathetic activity increased* with a concomitant increase in blood pressure and pulse frequency The result of one of the experiments is shown in Fig 3 Both smokers inhaled the smoke and although it is not very obvious in the Figure the respiration changed slightly in character during smoking which may have contributed to the rather high intensity of some of the individual sympathetic bursts The effect of smoking was not confined to the actual smoking period but both the increase in sympathetic nerve activity and the blood pressure response were rather long lasting and in the case shown in Fig 3 the pre smoking level was not reached until 3 min after the smoking was ended

Changes in body position Valsalva s manoeuvre
As shown in a preceding paper (Delius *et al* 1971 b) postural changes from the horizontal to a more vertical position elicited a characteristic increase in sympathetic

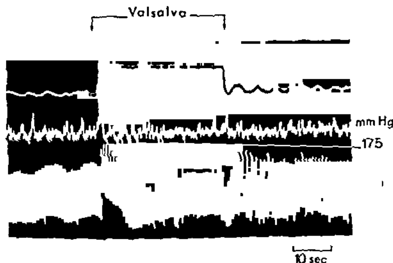


Fig 4 The effect of Valsalva's manoeuvre on sympathetic activity recorded in a median nerve fascicle innervating glabrous skin Same tracings as in Fig 1, 2 and 3

muscle nerve activity indicative of a reduced inhibitory influence from the baroreceptors The same test was repeatedly performed when recording from skin nerves but in contrast to the finding in muscle nerves the sympathetic skin nerve activity generally did not change in a significant way Sometimes the activity did indeed increase during the tilting procedure but as the effect was transient and occurred independently of the direction of movement the reaction was interpreted as an unspecific response (see Discussion) In one subject there was a reproducible decrease of sympathetic skin nerve activity in the standing position but in the majority of cases no change was noted

During *Valsalva's manoeuvre* the mechanically elicited decrease in venous return leads to a fairly pronounced reduction of the systolic blood pressure In muscle nerves the blood pressure reduction was accompanied by an increase in sympathetic activity, supporting the concept of a baroreflex control of the sympathetic outflow to skeletal muscle (Delius *et al* 1971 b) The response was quite different in skin nerves and Fig 4 shows a typical example of the effect of Valsalva's manoeuvre on sympathetic skin nerve activity The manoeuvre was performed 18 times by 6 subjects and apart from one or a few initial strong sympathetic bursts (probably triggered by the deep inspiration) the spontaneous skin nerve activity usually continued more or less unchanged in spite of the pronounced variations in blood pressure In good agreement with this finding no change in skin vascular resistance was observed during the period after the Valsalva manoeuvre The results indicate that sympathetic outflow to the vascular bed of the skin is not involved in compensatory reactions during changes of arterial blood pressure

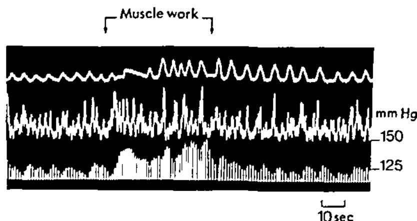


Fig 5 The effect of muscle work (isometric hand contraction) on sympathetic activity recorded in a peroneal nerve fascicle innervating hairy skin Same tracings as in Fig 1 2 3 and 4

Muscle work

Periods of fairly intense isometric muscle contractions were performed on 12 occasions in 6 subjects while recordings were made from a skin nerve in an extremity not engaged in the work In 6 experiments on 3 subjects this resulted in a slight increase in sympathetic skin nerve activity whereas no change was observed during the remaining 6 expts Fig 5 shows an example from one of the 3 subjects where a simultaneous i a blood pressure recording was made During the period of muscle contraction a slight increase of sympathetic skin nerve activity can be observed together with a clear increase in systolic blood pressure It should be noted that the contraction period usually was associated with an increase in both the depth and frequency of respiration

Hyperventilation

As discussed by Hagbarth *et al* 1971 a sudden deep breath was an effective way of eliciting a burst of sympathetic impulses the intensity of which usually exceeded that of the spontaneously occurring discharges Both during a sequence of deep breaths and during true hyperventilation most inspirations were accompanied by similar reflex responses leading to an increase in sympathetic skin nerve activity (Fig 6) The result was similar during 8 periods of hyperventilation whereas no change was noted in the 2 remaining experiments In the example shown in Fig 6 the neural outflow increased before the hyperventilation started indicating that an initial preparatory response was added to the specific effect of the hyperventilation When this initial effect subsided the neural response declined and the blood pressure fell

Discussion

Present results in comparison with previous neurophysiological findings in animals

The results of numerous investigations of the peripheral circulation indicate a highly

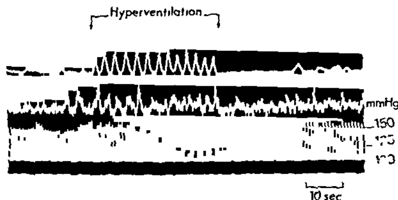


Fig 6 The effect of hyperventilation on sympathetic activity recorded in a median nerve fascicle innervating glabrous skin Same tracings as in Fig 1, 2, 3, 4 and 5 Note 'preparatory' bursts before the start of the manoeuvre

differentiated control of the sympathetic outflow to different vascular beds (for references see Mellander and Johansson 1968) On the other hand with a few recent exceptions (Walther, Iriki and Simon 1970 Iriki *et al* 1971), previous recordings of activity in pre- and postganglionic cervical, thoracic or abdominal sympathetic nerves have shown no signs of such differentiated regional control This unfortunate lack of agreement between neurophysiological and circulatory data seems to be partly eliminated by the present results where frequent examples of differences between sympathetic outflow in skin- and muscle nerves were found One main reason for the differences in results between previous neurophysiological investigations and the present work is probably that evidence for differentiated control of sympathetic outflow is hard to find in multiunit recordings from mixed sympathetic nerves close to the ganglia, where it is not possible to distinguish between fibres destined for various tissues and various types of effector organs Furthermore the present recordings were made on awake subjects, whereas many previous neurophysiological studies were made on animals under general anaesthesia, which affects the central regulatory mechanisms involved in the control of the sympathetic outflow

Conditional and unconditional sympathetic skin nerve responses

Previous measurements of sympathetic effector organ responses in awake subjects have demonstrated a high sudomotor and skin vasomotor reactivity to a variety of external and internal stimuli As emphasized by the Russian school (for references see Figar 1965) this high responsiveness to stimuli, inducing orienting defensive or thermoregulatory adaptive reactions, makes it hard to define a basal level of skin vasoconstrictor tone Also, conditional vasomotor responses to initially ineffective stimuli are easily established This agrees with our findings that the sympathetic outflow to the skin was enhanced by a variety of novelty stimuli and manoeuvres causing changes in the attentative or emotional state of the subject The transient

initial increase of sympathetic skin nerve activity often seen just before or at the start of a manoeuvre should probably be interpreted as an expectancy or orienting reaction rather than as a specific response to the test performed Sudden tilting manoeuvres for instance, or active muscle contractions were often accompanied by such 'unspecific' sympathetic responses in the skin nerves

As discussed by Delius *et al* (1971 b), the sympathetic mass discharges recorded from skin nerves may be composed of both vasoconstrictor and sudomotor impulses and as a rule it is difficult to assess their relative contribution to the neural activity It seems likely, however that both fibre groups contributed to the increased sympathetic outflow during manoeuvres involving mental strain or orienting and defensive reactions whereas body cooling with reasonable certainty activated only vasoconstrictor and not sudomotor fibres

Sympathetic responses in skin and muscle nerves with accompanying vasomotor reactions

Table I summarizes our findings concerning the effect of various manoeuvres upon the sympathetic neural outflow to skin and muscles The table also includes data from the literature concerning vascular resistance changes in skin and muscles induced by the various tests (for references see Greenfield 1963 Barcroft 1963 Shepherd 1963) Our own determinations of vascular resistance during the nerve recordings agree with these previous findings

a) *Neural events compared to effector organ responses* The Table shows that for most of the manoeuvres we have found good agreement between neural and vascular events plus and minus variations in the neural outflow correspond to similar changes in vascular resistance Some exceptions from this rule were seen in our experiments however 1) Mental or emotional stress always induced an increase in the sympathetic outflow and usually cutaneous vasoconstriction but sometimes a tendency to vasodilatation was observed as has also been reported in the literature Local release of vasodilator substances from the sweat glands can probably explain such reactions 2) The relative sluggishness of the effector organ responses and/or difficulties in obtaining a sensitive continuous measure of muscle blood flow may explain why short pauses in the sympathetic outflow to the muscles like those induced by single deep breaths are not known to be accompanied by signs of muscle vasodilatation The high vascular resistance in skeletal muscle in spite of complete cessation of the sympathetic outflow during the relaxation phase after the Valsalva manoeuvre is another example of apparent disagreement in the Table which may be explained as due to effector organ sluggishness In this case however a myogenic vascular reaction induced by the blood pressure overshoot may also be involved in the maintenance of the high vascular resistance (Folkow 1964)

b) *Comparison of sympathetic responses in skin and muscle nerves* It is obvious that those manoeuvres (Valsalva changes of body position) which are known to elicit typical baroreflex responses (Sharpey Schafer 1953 Bridgen Howarth and Sharpey Schafer 1950 Roddie and Shepherd 1956 caused changes in sympathetic

(1963) and Shepherd (1963)

Manoeuvre	Changes of sympathetic activity		Changes of vascular resistance	
	Muscle	Skin	Muscle	Skin
Head up tilting	+	0(±)	+	0(±)
Raising the legs	−	0	−	0
Valsalva (during)	+	0	?	?
Valsalva (after)	−	0	+	0
Mental arithmetic	−	+	−	+(−
Muscle work	+	+, 0	+	+
Deep breath	−	+	0	+
Hyperventilation	−	+	−	+
Cooling	0	+	0	+
Warming	0	−	0	−
Smoking	0	+	0?	+

outflow to skeletal muscles but not to the skin The reverse was seen in response to thermal stimuli. This corresponds to the functional differentiation between skin and skeletal muscle vessels, with skin vessels engaged mainly in thermoregulation and muscle vessels in circulatory homeostasis

During mental arithmetic we found opposite sympathetic responses in skin and muscle nerves, corresponding to the well known simultaneous constriction in skin and dilatation in muscle vessels It remains for further experimentation to clarify whether this was due to a primary central reciprocity or whether the inhibitory reaction in muscle nerves causing vasodilatation was a secondary effect elicited by the blood pressure elevation via the baroreflex (cf Delius *et al* 1971 b) Recent animal studies of interactions between hypothalamic defence reactions and baroreflexes suggest that both mechanisms may be involved (Djojosugito *et al* 1970)

Hand vasoconstriction following a single deep breath was reported long ago (Bolton, Carmichael and Sturup 1936) and as expected this test caused reflex activation of sympathetic impulses to the skin (Hagbarth *et al* 1971) The finding was confirmed in the present experiments where increased respiratory movements regularly increased sympathetic skin nerve activity When recording sympathetic muscle nerve activity on the other hand both single breaths and voluntary hyperventilation inhibited the sympathetic impulses Delius *et al* 1971 a and b) indicating that not only the local effect of an altered pCO (Burnum Hickam and McIntosh 1954 Roddie, Shepherd and Whelan 1957) but also reflex mechanisms participate in skeletal muscle vasodilatation The effect of smoking on sympathetic skin nerve activity may partly be due to the altered respiration but in addition a pharmacological, neurally mediated vasoconstrictive effect seems likely

Muscle work was regularly accompanied by an increased sympathetic outflow to the muscle not engaged in the work, whereas the outflow to the skin was either uninfluenced or slightly increased It is hard to tell whether the latter effect was an unspecific 'arousal' response, a specific reflex response (induced from receptors in the working muscles) or a response secondarily induced by alterations in the respiratory movements This illustrates the fact that some of the manoeuvres involve many different sensory and behavioural stimuli which all tend to interact in a complex pattern, where the final vasomotor effect of each manoeuvre depends on the relative strength of the various interacting influences

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Measurement of Blood Flow in Adipose Tissue from the Washout of Xenon-133 after Atraumatic Labelling

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Abstract

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The experiments were performed on isolated, autoperfused subcutaneous tissue in urethane-

techniques

Measurement of regional circulation by external monitoring of the disappearance rate of radioactive isotopes injected directly into tissues was introduced by Kety (1949)) using radioactive sodium in skeletal muscle. Kety's principle was applied to the measurement of blood flow in skeletal muscle using radioactive Xenon-133 dissolved in saline (Lassen, Lindbjerg and Munck 1964, Holzman *et al.* 1964). Studies of washout of lipid soluble molecules indicate that capillary to tissue diffusion is rapid and that blood flow is the rate-limiting factor in transport. Renkin 1952) suggesting that virtually complete equilibrium of these gases takes place between tissue and blood during a single passage through the capillary bed. Direct measurement of the tension of inert gases in the tissue and the effluent blood during desaturation has never been made but indirect evidence for diffusion equilibrium during the washout of the tracer can be obtained if blood flow values measured by this technique agree with simultaneous measurement using other reliable methods (Renkin 1955). Such studies have been performed on skeletal muscle (Sejrsen and Tonnesen 1968).

Assuming diffusion equilibrium between adipose tissue and blood during the was

out of the radioactive inert gases, Larsen, Lassen and Quaade (1966) applied the local Xenon-133 washout technique to measurement of blood flow in the abdominal subcutaneous adipose tissue in man Following an initial fast washout rate ascribed to injection trauma, the washout curve was found to be monoexponential as long as 48 h after the injection, when 99 per cent of the tracer had been cleared If the adipose tissue could be labelled atraumatically only a monoexponential washout curve for the tracer should be observed, indicating that adipose tissue is a homogeneous washout system (Larsen and Lassen 1967)

The present work has attempted to measure the washout curve following atraumatic introduction of Xenon-133 in adipose tissue and to compare the calculated values of blood flow per unit tissue weight for the locally labelled area with the directly measured outflow of blood from an entirely isolated preparation of adipose tissue

Experimental procedure

Adipose tissue preparation The experiments were performed on 10 rabbits (white Danish country breed) weighing 2 9 to 4.3 kg After being fasted for 12 hrs the animals were anesthetized intravenously with urethane (ethylcarbamate), 1 0 gram per kg body weight Tracheostomy was performed and the animals' rectal temperature was kept at 37° C

The subcutaneous adipose tissue preparation was carried out as a modification of the technique described by Orö *et al* (1965) as used recently by Lewis and Matthews (1968) The subcutaneous adopose tissue in either the right or left inguinal region was exposed To minimize operative trauma to the adipose tissue, the loosely attached skin was separated from the [illegible]derlying adipose tissue by sharp dissection and ligation of all connecting vessels and the [illegible] was covered by a polythylene sheet to avoid drying during the preparation The fat pad was exposed as far laterally as possible, tied and divided where it became thin The tissue was also divided between ligatures cranially and caudally The cranial ligatures isolated the pad from the mammary glands while the caudal ligatures were placed just below the entrance of the superficial epigastric vessels The adipose tissue preparation weighing between 5 5 and 26 1 g (mean 13 0 g was covered by moist gauze and a polyethylene sheet The temperature monitored by a thermocouple placed on the surface of the adopose tissue, was kept at 37° C by a heating lamp

Following heparin 10 mg/kg i v) the femoral vein was isolated for about 4 cm below the inguinal ligament and all side branches except the superficial epigastric vein were ligated After ligation of the femoral artery the femoral vein was cannulated distal to the superficial epigastric vein The venous outflow from the fat pad was measured using a photoelectric drop counter The system was calibrated using a stop-watch and a graded reservoir The blood was returned to the animal through a catheter in the external jugular vein Left common carotid arterial pressure and venous outflow pressure were monitored using Statham strain gauge transducers All parameters were recorded on a 4-channel Beckman Dynograph type R Care [illegible] adipose tissue during its surgical isolation [illegible]ur Xenon 133 in gaseous form was obtained from [illegible] Xenon 133 in isotonic saline (5–10 mCi/ml from [illegible]ngland With the atraumatic technique described by Sejrsen (1968 the radioactive gas was applied t the adipose tissue The surface of the adipose tissue was moistened with isotonic saline and covered with a 20 μm polyethylene membrane to minimize the evaporation and damage due to trauma A plexiglas cup (10 mm inner diameter and 1 mm inner height placed at my disposal by Dr P Sejrsen) was gently pasted to the membrane with a drop of isotonic saline Through a thin injection needle leading into the chamber 0 3 ml gaseous Xenon 133 was injected from a syringe To maintain ambient atmospheric pressure within the chamber during the labelling period a vent was created using a fine, 70 mm long catheter After 2–3 min the Xenon 133 gas was redrawn into the syringe, the chamber was removed and surplus of the gas was blown away The labelled area of the adipose tissue was covered by a 20 μm thick gastight Mylar membrane which was sealed to the polyethylene membrane by a drop of water The external recording of the [illegible] emission

from the Xenon 133 depot was started 1 minute after the end of the labelling period A one inch NaI (Tl) crystal was placed about 10 cm above the fat pad and collimated so as to see the whole of this The detector was connected to a one channel analyzer (Philips PW 4280 set at a narrow window to record only the 81 keV radiation of Xenon 133 The pulses were fed into a double scaler (Meditronic Denmark) alternating each minute, with a counting time loss of 10 microsecond per period This double scaler operated a digital printer (Modu print A 3—84) so that counts were recorded for periods of one minute To maintain low back ground the room was well ventilated The counting over the Xenon-depot was performed for 50 to 210 min depending on the stability of the directly recorded blood flow The superficial epigastric vessels were then clamped, and measurement was continued for an additional 20 min period to assure that neither venous flow to the dropchamber from sources other than the epigastric vein nor non vascular escape of Xenon 133 from the depot had occurred during the study

After subtraction of the background, the counts were plotted on semilogarithmic paper against time to determine whether the results could graphically be fitted by a monoexponential function when the directly recorded blood flow was constant

Analytical methods

In all experiments hematocrit values were determined repeatedly on arterial blood In the last seven experiments the concentration of hemoglobin plasma protein and plasma lipid and the composition of the adipose tissue was determined by the following methods

(1) Arterial hemoglobin concentrations were determined by analysis of cyanohemoglobin (Borovicczeny 1964)

(2) Total plasma protein was determined from the specific gravity of plasma (copper sulphate method)

(3) Total plasma lipid was estimated using the extraction procedure of Folch *et al* (1957), followed by weighing the residue after drying

(4) Lipid content of the adipose tissue [illegible] from the labelled area of the fat pad The samples, weighing a [illegible] Elvehjelm homogenizer and subsequentl[illegible] After separation and removal of non lip[illegible] determined by weighing and the content of lipid were then expressed in per cent of the wet tissue weight

(5) Water content of four samples of adipose tissue from the labelled area of the fat pad was determined by weighing the tissue before and after drying to constant weight in an exsic cator at 40—50° C The figures reported are the average of the four values obtained in each experiment

Calculations

The desaturation curve for Xenon 133 from the subcutaneous adipose tissue was treated as a monoexponential function applying a monocompartmental model Thus Kety's classical monoexponential clearance function

$$(C(t) = C(O) \exp [(-f/\lambda) \quad t]$$

was used in the calculations where f stands for blood flow rate per unit weight of tissue A number of conditions must be fulfilled for this approach to be valid The major assumptions are homogenous distribution of tissue perfusion and diffusion equilibrium for the tracer be tween tissue and capillary blood at any time during the washout Thus blood flow becomes the only rate limiting factor to disappearance of tracer from the tissue and tension gradients are assumed not to exist in the labelled area In addition shunts lymph flow, counter-current mechanisms (Sejrsen and Tønnesen 1968) and intercompartmental diffusion processes (Perl 1962, Sejrsen 1967) should have only minor influence on the removal of tracer from the tissue The labelled area should be representative of the whole tissue Furthermore the parti t on coefficient (λ) must be known from separate determinations The recirculation of Xenon 133 to the field seen by the detector must be negligible This condition is unquestionably ful filled since recirculation amounted to only about 1 per cent when intra arterial injection was used in studies of the isolated gastrocnemius muscle (Tønnesen and Sejrsen 1967) This preparation received a larger fraction of cardiac output and showed a faster washout tracer than adipose tissue

It follows from the Kety equation that

$$ATBF = k \cdot \lambda \cdot 100 \ (ml/100 \ g \ min)$$

where ATBF is the adipose tissue blood flow, λ is the tissue to blood partition coefficient for Xenon 133 at equilibrium, and k is the rate constant of the washout curve

The slope of the regression line for the logarithmic transformed count figures on time was calculated in each experiment by the method of least squares The rate constant is the numerical value of this slope

The value of the partition coefficient was in seven experiments estimated from the above mentioned chemical analyses of the blood and the adipose tissue and the following Ostwald solubility coefficients (ml gas STP/g at 37° C) for Xenon 133 given by Yeh and Peterson (1963, 1964 1965) in isotonic saline (0 0778), in human albumin (0 1493), in human hemoglobin (0.3661) and in human fats (1 8424) Components in blood other than hemoglobin, plasma protein, and plasma lipid are assumed to possess a solubility for Xenon equal to that of isotonic saline The same assumption was made regarding components in adipose tissue other than the lipid fraction No correction was made for the small amounts of phospholipid in tissue and blood, nor for the blood content in the tissue

Results

The composition of blood and adipose tissue at the end of the experiments is shown in Table I (seven experiments on six rabbits) Using the average figures for blood and tissue components and the appropriate values for Xenon solubility in the respective substances, the mean value of the partition coefficient was calculated to be 11 8 (ml/g) The rather low hemoglobin concentration and the great variation in lipid rcentage in adipose tissue of rabbits will be discussed

Fig 1 shows a typical washout curve of Xenon 133 from adipose tissue after atraumatic labelling The disappearance curve of the tracer is well fitted by a straight line in the semilogarithmic diagram ($r = 0\,999$, $p < 0\,001$) In preliminary experiments where Xenon 133 dissolved in isotonic saline was applied by gentle local injection of 0 1 ml through a fine needle (o diam 0 4 mm) or where Xenon 133 in gaseous form was applied without a gas permeable polyethylene sheet against the tissue, an initial faster disappearance rate was seen This 'trauma-phase' lasted 10—30 min

Direct recording of the adipose tissue blood flow was compared with the blood flow calculated from the Xenon 133 washout rate in 11 expts The results are shown in Table II The weight of the adipose tissue at the end of the experiments ranged from 5 5 to 26 1 g Effluent venous pressure was kept between 0 and 5 mm Hg throughout the experiments and in two experiments the increase in tissue water was found to be less than 1 5 % when compared to the contralateral subcutaneous adipose tissue The directly measured blood flow was recorded in periods of 5 min. and at the end of the experiment the flow in ml 100 g min was calculated The values shown in Table II is the arithmetic mean value and the fluctuation of the blood flow recorded in this way is expressed by the standard deviation In rabbit no 8 a sudden change occurred in blood flow 50 min after the labelling simultaneous with a fall in blood pressure but thereafter flow remained constant for 60 min (The results obtained in each of these periods are reported here as exp 8 and 9)

TABLE I Composition of blood and subcutaneous adipose tissue in experimental rabbits

Experiment No	Rabbit No	Hematocrit ml/100 ml	Hemoglobin g/100 ml	Plasma-protein g/100 ml	Plasma lipid g/100 ml	Water g/100 ml	Subcutaneous adipose tissue		
							Lipid g/100 g	Water g/100 g	λ* ml/g
5	5	32 3	11 5	5 6	0 56	83 1	73 7	26 3	11 0
6	6	29 9	9 2	5 5	0 70	83 7	84 6	27 1	13 0
7	7	29 8	10 8	5 5	0 48	83 8	90 6	9 6	13 9
8	8	34 4	10 1	6 0	0 54	83 3	62 7	39 8	9 8
9	8	32 5	9 4	5 8	0 53	83 6	62 7	39 8	10 1
10	9	31 1	8 9	5 9	0 44	83 4	67 5	32 4	11 1
11	10	35 2	11 3	6 2	0 52	82 1	90 2	9 7	13 5
Mean		32 2	10 2	5 8	0 54	83 3	76 0	26 4	11 8
S D		2 1	1 0	0 3	0 08	0 6	12 4	12 6	1 7

* The adipose tissue to blood partition coefficient (λ) for Xenon 133 has been calculated according to Yeh and Peterson (1965)

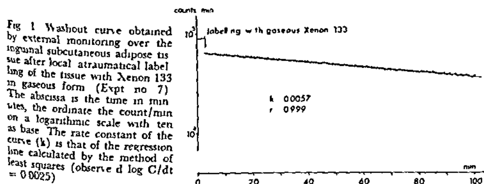


Fig 1 Washout curve obtained by external monitoring over the inguinal subcutaneous adipose tissue after local atraumatical labelling of the tissue with Xenon 133 in gaseous form (Expt no 7) The abscissa is the time in minutes, the ordinate the count/min on a logarithmic scale with ten as base The rate constant of the curve (k) is that of the regression line calculated by the method of least squares (observe d log C/dt = 0 0025)

The computed rate constant of the Xenon 133 washout in each experiment is also listed in the table together with the regression coefficient for the washout curve The regression coefficients indicate the validity of the monoexponential approach for describing the tracer washout from the tissue in the time period of the study The amount of tracer cleared from the tissue in this period averaged 37 per cent (range 9 to 63 per cent)

In the last columns the values of blood flow in adipose tissue calculated from the washout curves by means of an average value for the partition coefficient (I) and the value measured in each experiment (given in Table I) (II) are shown For the last seven experimen (no 5—11) the mean values of blood flow calculated in these ways were respectively 10 % and 4 % higher than the average of the directly recorded blood flows The differences between the calculated values are mainly due to the two different estimations of lipid contents in the adipose tissue Fig 2 illustrates the relation between the calculated adipose tissue blood flow and the directly me-

TABLE II Directly recorded adipose tissue blood flow (ATBF) in comparison to numerical analysis of the Xenon 133 washout curve

Experiment No	Rabbit No	Adipose tissue weight g	ATBF directly recorded		Computed slope			ATBF calculated* ml/100 g min	
			ml/100 g min	S D	k	r	p	I	II
1	1	21.7	6.8	0.1	0.0069	0.9978	< 0.001	8.1	—
2	2	11.1	5.4	0.6	0.0046	0.0088	< 0.001	5.4	—
3	3	26.1	2.3	0.2	0.0023	0.8318	< 0.001	2.7	—
4	4	18.3	4.9	0.4	0.0044	0.9870	< 0.001	5.2	—
6	5	15.0	5.8	0.3	0.0050	0.9923	< 0.001	5.9	5.5
6	6	8.1	2.9	0.3	0.0026	0.9919	< 0.001	3.0	3.4
7	7	10.4	6.6	0.7	0.0057	0.9989	< 0.001	6.7	7.9
8	8	5.5	17.1	1.3	0.0175	0.9976	< 0.001	20.7	17.2
9	8	5.5	10.9	0.9	0.0112	0.9938	< 0.001	13.2	11.3
10	9	7.3	7.0	0.5	0.0063	0.9575	< 0.001	7.4	7.0
11	10	13.1	3.4	0.1	0.0027	0.9763	< 0.001	3.2	3.6
Mean		12.9	6.6					7.4	
S D		6.8	4.2					5.3	

* The λ value used is (I) the average obtained from exp no 5—11 (11.8 ml/g) and (II) the value from the single experiment (Table I)

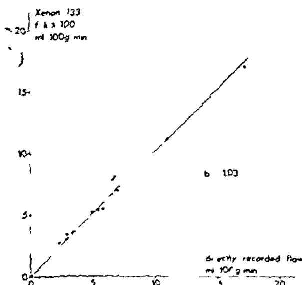


Fig 2 Comparison between the directly recorded blood flow and the blood flow calculated from the washout curves (see Table II) The triangles and the open circles are respectively the experiments 1—4 and 5—11 both calculated using an average λ value of 11.8 ml/g The dots denote the experiments number 5—11 calculated from the λ-value determined for each experiment, with the calculated regression line shown in the figure

ured blood flow Including only those experiments where individually determined λ-values were used for flow calculations the equation for the regression line ($r = 0.995$, $p < 0.001$) determined using the method of least squares was calculated to be $y = 0.99x + 0.41$ The intercept did not differ significantly from zero, and therefore it was assumed that the true line passed through zero According to this the

slope was calculated to be 1 03, $S_b = 0\ 23$, indicating that the slope is not significantly different from unity ($p > 0\ 30$) Thus, there was no systemic difference between the values of adipose tissue blood flow observed with direct recording and with Xenon 133 washout techniques

Discussion

In general, the Xenon 133 washout technique gives reliable values for blood flow only if diffusion equilibrium between the tissue and the capillary blood exists throughout the experimental period, as was theoretically deduced by Copperman (quoted by Kety 1951) from calculation of diffusion in a tissue cylinder Experimental evidence of the existence of diffusion equilibrium between adipose tissue and effluent blood for the radioactive, inert gases was first obtained in double tracer experiments by Larsen, Lassen and Quaade (1966), who found agreement between the ratio of washout constants and partition coefficients for Xenon 133 and Krypton-85 in human subcutaneous tissue Further and more decisive evidence has been shown in the present study by the close agreement between the directly recorded flow and the flow calculated from Xenon-133 washout over the whole range of flow values (Renkin 1955)

The applicability of a monocompartmental model for the washout of the inert gases from adipose tissue is supported by the Xenon-133 washout curves observed after atraumatic labelling of the tissue by diffusion of the gas from the surface into the gently isolated tissue The washout curves obtained in this way in skeletal muscle are representative for washout curves after equilibrium of the entire tissue with the tracer (Sejrsen and Tonnesen 1968) The washout curves all fitted a monoexponential function throughout the study when the blood flow through the adipose tissue was constant The amount of Xenon 133 cleared monoexponentially after atraumatic labelling during this study averaged 37 per cent Washout curves of Xenon 133 from subcutaneous tissue in man after local injection has been followed over two decades (Larsen, Lassen and Quaade 1966) and was found monoexponential after initial, fast washout of about 5 per cent of the tracer The initial, fast part of these curves must be ascribed to traumatic effects of the injection Without these it is likely that the washout curves would be monoexponential during the entire washout process Discrepancy from the model be it heterogeneity in regional perfusion or in inert gas solubility over macroscopic distances diffusional limitation in transport and countercurrent mechanisms, would all tend to cause nonlinearity of the washout curves However small deviations might be masked due to the very low fractional loss of tracer per unit time from adipose tissue A differentiation of the desaturation curve is obtainable in an experiment performed with external monitoring after intra arterial bolus injection Such experiments are necessary to unmask finer details in the washout conditions

Adipose tissue is known to be well vascularized (Gersh and Still 1948) The agreement between the directly recorded blood flow and the values achieved from

TABLE III Values of adipose tissue blood flow (ATBF) collected from the literature Perfusion with other fluids than blood has not been included In studies with ¹³³Xenon and ⁸⁵Krypton as tracer results have been recalculated with a partition coefficient on 10 0 and 7 5 *respectively making results comparable*

Author		Animal	Adipose tissue	Technique		ATBF (ml/100 g min)
				Tracer	Registration	
Lundin	1960	Human	Total body	N_2	washout after inhalation	2 5
Perl et al	1960	Human	Total body	Cyclopropane	uptake during inhalation	3 1
Larsen et al	1966	Human	Inguinal subcutaneous	¹³³Xe	ext monitoring after local injection	2 6
Lesser and Deutsch	1967	Human	Total body	⁸⁵Kr	ext monitoring during inhalation	2 1
Sejrsen	1967	Human	Lower extr subcutaneous	⁸⁵Kr	ext monitoring after bolus injection	3 0
Oro et al	1965	Dog anest	Inguinal subcutaneous	—	direct outflow	6 7
Lewis and Matthews	1968	Rabbit *anest*	*Inguinal subcutaneous*	—	*direct outflow*	8 1
Nielsen	1971	Rabbits anest	Inguinal subcutaneous	—	direct outflow	6 6
Nielsen	1971	Rabbits anest	Inguinal subcutaneous	¹³³Xe	ext monitoring after local appl	6 3
Mayerle and *Havel*	1969	Rats anest	Epididymal	⁸⁶Rb	fractional uptake after bolus injection	5 3
Herd et al	1968	Rats unanest	Inguinal subcutaneous and epididymal	DDT	uptake after saturation	10 0
Tonnesen	pers com	Seal		¹³³Xe	ext monitoring after local injection	7 0

the washout curves whereever the tissue was labelled implies that perfusion of the adipose tissue is homogeneous not only in the *labelled* region, but *also in the adipose tissue preparation* as a whole

When the data in Table I are used to calculate the partition coefficient for Xenon 133, as proposed by Yeh and Peterson (1965) the value is higher than the values previous published (Conn 1961 Andersen and Ladefoged 1967) However the overwhelming importance of the contents of lipid in the tissue should be emphasized If the contents of lipid in rat adipose tissue as given by Andersen and Ladefoged (1967), is used for calculation of the partition coefficient with a normal composition of blood (Handbook of biological Data 1956) and a hemoglobin concentration on 15 g/100 ml the calculated value becomes 9 2 ml/g This is in agree

ment with the value of 9 8 g/g S D 2 3 experimentally determined by Andersen and Ladefoged (1967)

The use of an average value for the partition coefficient in man requires uniform composition of tissue and blood Larsen, Lassen and Quaade (1966) used a value of 10 0 ml/g for the subcutaneous adipose tissue on the lower part of the abdomen If however the partition coefficient is calculated from a normal composition of abdominal subcutaneous adipose tissue (90 3 % (w/w) lipids and 8 9 % (w/w) water (Martinsson 1967), and a normal composition of blood with hematocrit 40 % (Handbook of biological Data, 1956), the value of the adipose tissue to blood partition coefficient becomes 12 0 ml/g The disagreement in recent literature between the values for the partition coefficient for Xenon 133 in adipose tissue indicates that further studies on this topic are necessary

Table 3 presents values for adipose tissue blood flow collected from the literature noting animal species tissue location and techniques of blood flow measurement The values for the adipose tissue blood flow achieved from the washout of inert gases are 2—3 ml/100 g min in man, while the directly recorded blood flow in subcutaneous adipose tissue in dog and rabbit is 6—8 ml/100 g min This discrepancy is only partly explained by the uncertainty in the determination of the partition coefficient. The influence of animal species location of adipose tissue and nutritional and neurohormonal state of the animal on the blood flow in adipose tissue still remains to be elucidated (*cf* Fredholm 1970) The significance of the tissue location (Herd *et al* 1968) and the number of fat cells per unit tissue weight (Girolamo *et al* 1971) on adipose tissue blood flow has recently been emphasized

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Reflex Relaxation of the Stomach Elicited from Receptors Located in the Heart. An Analysis of the Receptors and Afferents Involved.

By

HASSE ABRAHAMSSON and PETER THORÉN

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Abstract

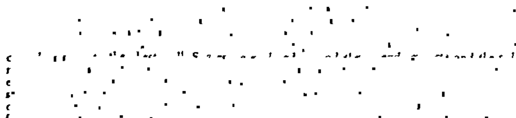
ABRAHAMSSON, H and P THORÉN *Reflex relaxation of the stomach elicited from receptors located in the heart* An analysis of the receptors and afferents involved Acta physiol scand 1972 *84* 197—207

In cats anesthetized with chloralose electrical afferent stimulation of the right cardiac nerve promptly elicited marked hypotension bradycardia and relaxation of the stomach. Stimulation threshold and conduction velocity measurements indicated that the stimulated afferents were unmyelinated fibres The circulatory and gastric responses were abolished by cervical vagal blockade on the stimulation side Administration of nicotine intrapericardially or veratrum alkaloids i v produced similar circulatory and gastric responses as the electrical nerve stimulation The effects of veratrum were markedly reduced upon application of a local anesthetic in the pericardial cavity Reflex gastric relaxation was also elicited in response to occlusion of the ascending aorta or mechanical stimulation of the left ventricle With occlusion of the pulmonary artery only slight gastric responses were obtained during the occlusion Pronounced gastric responses were recorded upon occlusion of a coronary artery, the effect being abolished by section of the vagi in the neck — It is concluded that marked reflex relaxation of the stomach can be elicited from heart receptors probably located in the left ventricle and with unmyelinated afferents in the vagi It is suggested that the described reflex gastric response may form part of a vomiting pattern

Receptors located in the heart have been supposed to be involved in circulatory adjustments occurring during various pathophysiological situations like myocardial infarction (Costantin 1963) and in the vasovagal syncope reaction (Pearce and Henry 1955, Öberg and White 1970 b, Öberg and Thoren 1970) Such circulatory conditions are known to be often associated with nausea and various gastrointestinal symtoms like vomiting Similar gastrointestinal disturbances occur with administration of veratrum alkaloids which are known to stimulate heart receptors From such observations it seemed to be of interest to analyse whether, and then to what extent, heart receptors are capable to reflexly influence gastrointestinal motility In the present study the effects of heart receptor stimulation on gastric motility was analysed in anesthetized cats

Methods

Experiments were performed on 15 cats (weight 2.3—4.0 kg) deprived of food for 24—36 h before the experiment. The animals were anesthetized with chloralose 40—50 mg/kg b.w. after



ventional way via an amplifier on an oscilloscope and photographed. In three cats a snare was placed around the anterior descending coronary artery to allow intermittent occlusions.

In some of the experiments the vagal impulse traffic could be blocked temporarily by cooling the vagi in the neck. This was done by placing the nerves on probes which could be cooled down to 0° C by perfusing them with a mixture of ice water and alcohol.

Arterial blood pressure was measured from the left subclavian artery by means of a Statham pressure transducer (P 23 AC) connected to a Grass Polygraph recorder, heart rate being monitored by a tachograph on the same recorder. The tachograph was triggered by the rapid systolic pressure rise in the arterial pressure curve.

Alterations of gastric motility was monitored as earlier described in detail by Jansson (1969). This technique implies a recording of gastric volume at a constant low intragastric pressure (4—8 cm H_2O) by means of a large rubber balloon introduced into the stomach via the esophagus. The balloon was connected via a large volume reservoir to a float recorder writing on a kymograph.

Reflexes from heart receptors were elicited in several ways. In some experiments the cardiac nerve was directly stimulated with square wave pulses (1—4 V, 0.01—2 ms) and at varying frequencies. In other experiments the receptors were stimulated mechanically by stroking the exposed epicardial surface with a plastic rod or by elevating the respective intraventricular pressure by tightening the snares around the aorta or the pulmonary artery. The receptors were stimulated pharmacologically either by nicotine, 5—200 µg dissolved in saline and injected into the pericardial cavity, or by protoveratrine (Puroverin® Sandoz), 5 µ i.v. Nicotine 5—50 µg was also injected i.v. After intrapericardial drug application the cavity was rinsed with isotonic saline. In some experiments a local anesthetic (Lidocain Xylocain® Astra 0.5 % 1 ml) was injected into the pericardial cavity.

Attempts were also made to activate the heart receptors by producing such pathophysiological circulatory situations as have been supposed to activate cardiac receptors in man. Thus, in four experiments the anterior descending coronary artery was obstructed temporarily to mimic an acute infarction. In other experiments the animals were rapidly bled from a catheter introduced in one of the femoral arteries in attempts to mimic a vasovagal syncope.

Results

I. Response to electrical stimulation of cardiac vagal afferents

The effect of cardiac nerve stimulation (10 imp/s, 1 V, 1 ms) on blood pressure, heart rate and gastric volume is illustrated in the left part of Fig. 1. The response consists of a blood pressure fall, an intense slowing of the heart (cf. Öberg and White 1970 a) and a pronounced relaxation of the stomach. The heart response appeared after a latency of 1—2 s and the gastric response within 1—5 s after the onset of stimulation. The pronounced circulatory responses and relaxations of the stomach appearing with such a short latency was observed in all experiments in this series. The right panel in Fig. 1 shows that clearcut circulatory and gastric responses to cardiac nerve stimulation are obtained with very low stimulation frequencies, usually already at 1—2 imp/s. The reflex responses increased in magnitude with increasing frequencies and reached a maximum at 10—15 imp/s with regard to the cardiovascular responses,

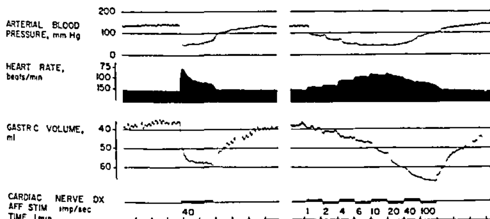


Fig 1

Left panel The effect of afferent stimulation of the right cardiac nerve (4 V, 1 ms) on arterial blood pressure, heart rate and gastric volume Note the "escape" of the heart and blood pressure responses during continued stimulation while the gastric response is well maintained during the stimulation period

Right panel Clearcut circulatory and gastric responses are obtained already at stimulation frequencies as low as 1—2 imp/s Maximal circulatory effects are obtained at 10 imp/s while the gastric response shows a further increase with stimulation frequencies up to 40—100 imp/s

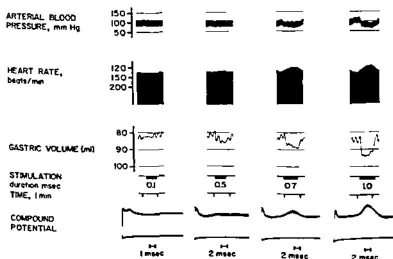


Fig 2 Circulatory and gastric responses to afferent stimulation of the right cardiac nerve (40 imp/s, 4 V) with increasing impulse durations The evoked compound action potential is

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while the gastric response become maximal first at a definitely higher frequency range, often first at 50—100 imp/s The maximal gastric response then obtained amounted to about a doubling of the initial volume, i e some 30 ml

Cutting of the right vagus nerve caudad to the stimulated right cardiac nerve did not eliminate the gastric response, indicating that the response was not due to any axon "reflex If, however, the right vagus was cut in the neck both the gastric and circulatory responses to cardiac nerve stimulation were totally abolished This indicated that the effect is of reflex nature and that the afferent pathway is in the vagal nerves

In the experiment illustrated in Fig 2 the evoked compound action potential was recorded from the stimulated cardiac nerve (4 V, 40 imp/s) proximally to the stimulation site When slowly increasing the impulse duration from low values up to 0 5 ms a 'late' component appeared in the neurogram and at the same time small but clearcut circulatory and gastric reflex responses were obtained This late evoked potential component was shown to have a conduction velocity between 0 6—1 3 m/s and must therefore reflect activity in unmyelinated C fibres With no exception the reflex gastric responses appeared at the same threshold value as the circulatory reflex effects and in all experiments this threshold value was of the same order of magnitude as shown in Fig 2 Consequently, the reflex circulatory and gastric responses shown in Fig 1 must be due to stimulation of unmyelinated afferents in the cardiac nerve

I Mechanical stimulation of heart receptors

Fig 3 panel A shows the effect of partial aortic occlusion on gastric volume heart rate and arterial blood pressure With a short latency there is a reflex bradycardia and a relaxation of the stomach which as a maximum correspond to 20 ml volume increase The reflex gastric response appears before the systemic blood pressure is reduced below 100 mm Hg as a result of the aortic obstruction which seems to exclude the possibility that a chemoreceptor reflex or regional gastric ischemia would be the cause of the gastric relaxation The aortic occlusion also evokes a pronounced reflex bradycardia Cooling of the cervical vagi completely eliminated the gastric relaxation and bradycardia during aortic obstruction Instead there was then rather a moderate tachycardia probably due to unloading of the arterial baroreceptors

As seen in panel B of Fig 3 occlusion of the pulmonary artery produces only slight gastric relaxation during the occlusion period but when the occlusion is released there is a moderate relaxation corresponding to maximally some 10 ml volume increase Similary heart rate is increased during the occlusion but after its cessation there is a steep rise in blood pressure and a bradycardia The described gastric responses were eliminated by cervical vagal cooling

Mechanical stimulation of the epicardial surface of the left ventricle by stroking with a plastic rod elicits a moderate gastric relaxation amounting to some 15 ml volume increase (Fig 3 panel C) Also this gastric response was eliminated by cooling the cervical vagi

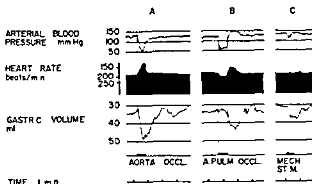


Fig 3 The effects of occlusion of the ascending aorta (A), occlusion of the pulmonary artery (B) and slight mechanical stimulation of the left ventricle with a plastic probe (C) Note that a reflex gastric relaxation and a reflex bradycardia occurs immediately when the aorta is occluded but with occlusion of the pulmonary artery first when the occlusion is released

III Reflex effects from heart receptors during coronary occlusion and rapid bleeding

The cardiovascular and gastric responses to occlusion of the anterior descending coronary artery was also studied as illustrated in Fig 4 Coronary artery occlusion for 1 1/2 min produces a decrease in heart rate and a blood pressure fall within 15—20 s after the onset of coronary obstruction and with about the same latency, a marked gastric relaxation appears After cessation of the occlusion the gastric volume slowly decreases again Fig 4 also shows that both the bradycardia and the gastric relaxation as a result of coronary occlusion are eliminated by cervical vagotomy, a small blood pressure response remains which is however probably a result of the direct ischemic effect on the heart interfering with its pumping action

The gastric response to a severe and rapid hemorrhage was studied in four cats Such a procedure can produce a pronounced reflex bradycardia of vagal origin (*e g* Öberg and White 1970 b) which is triggered from cardiac receptors (Öberg and Thoren 1970) The animal was first bled about 10 % of blood volume and two min later as fast as possible to a total of about 25 % reduction of the blood volume With this procedure it was possible to elicit a vagovagal bradycardia reflex Simultaneously with the reflex bradycardia a gastric relaxation amounting to some 40 ml volume increase was recorded This relaxation was reduced by cooling the vagi in the neck but still some relaxation was evident probably due to an effect via the splanchnics and adrenal medulla

IV Chemical stimulation of heart receptors

Nicotine injected in small doses in the pericardial cavity stimulates receptors in the epicardium of the left ventricle (Sleight 1964 Sleight and Widdicombe 1965 and reflexly elicits bradycardia and periferal vasodilation (Sleight 1964 Bergel and Makin 1966)

The circulatory and gastric response to small doses of nicotine (5—100 μg) injected in the pericardial cavity was examinated in 10 cats A typical response is seen in Fig 5 After a latency of 1—2 s there is a pronounced bradycardia and blood pressure fall, and within 4—6 s there is a marked gastric relaxation A clearcut g

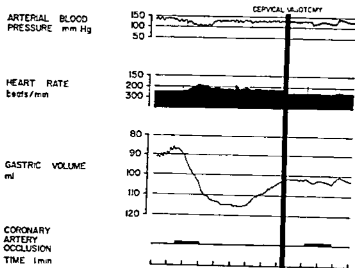


Fig 4 The effect of occlusion of the anterior descendent coronary artery on blood pressure heart rate and gastric volume Slowing of the heart and relaxation of the stomach appears simultaneously approximally 20 s after the onset of coronary occlusion No reflex responses to coronary artery obstruction is obtained after bilateral cervical vagotomy

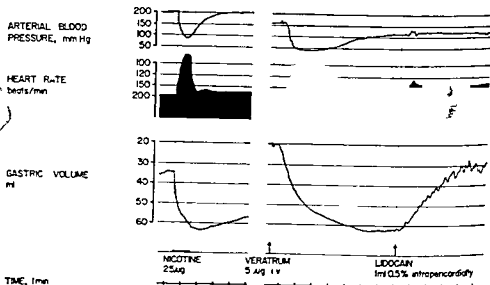


Fig 5 Pronounced hypotension brad cardia and gastric relaxation produced by local application of nicotine in the pericardium (left panel) and by intravenous injection of veratrum (right panel) The recovery of gastric volume after local administration of lidocain in the pericardium suggests that the relaxation caused by veratrum is elicited from receptors localized in the heart

response was elicited by only 5 μg nicotine and maximal effects were obtained with 100—200 μg The cardiovascular and gastric responses to epicardial application of nicotine could be repeated several times in each experiment and there was no sign of tachyphylaxis As with the other procedures described above cooling of the cervical vagi eliminated almost completely both the circulatory and gastric response to

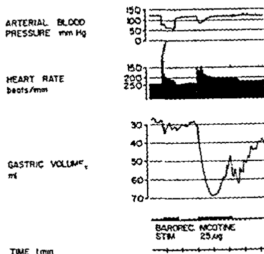


Fig. 6. Comparisons of the effect on blood pressure, heart rate and gastric volume of baroreceptor stimulation (pulling of both carotid arteries) and of nicotine injected locally into the pericardial cavity. Baroreceptor stimulation produces a more marked initial bradycardia and hypotension than intrapericardial nicotine application but essentially no gastric response.

nicotine. — As a control nicotine in doses of 25—50 μg were injected in the *pleural* cavity in two cats without any effects on circulation or gastric volume.

In four cats nicotine was injected *intravenously* in doses from 3—50 μg. In one cat where 5 μg nicotine produced a clearcut gastric response when injected intrapericardially the same amount given i.v. had no effect. 50 μg nicotine i.v. elicited a blood pressure rise but had no effects on gastric volume. It is therefore unlikely that the circulatory and gastric responses to nicotine when applied directly to the heart surface can be secondary to an absorbtion of the drug from the pericardial cavity.

Gastric responses to i.v. injections of veratrum alkaloids (Protoveratin® 5—20 μg) were studied in 3 cats. Results from 1 exp. in this series are illustrated in the right part of Fig. 5. After a latency of 20—25 s a pronounced bradycardia, blood pressure fall and a marked increase of gastric volume appears. Injections of a local anesthetic (Lidocain, 1 ml 0.5 %) into the pericardial cavity 2—7 min after the injection of protroveratrin led to a return of gastric volume towards control suggesting an interruption of a reflex activity.

I. *Comparison of the effects on gastric motility of baroreceptor stimulation and of heart receptor stimulation*

The circulatory and gastric responses to activation of cardiac afferents were compared with the effects produced by stimulation of the arterial baroreceptors. As illustrated in Fig. 6 pronounced baroreceptor activation (pulling the carotid arteries) causes intense bradycardia and hypotension which are more pronounced than those produced by nicotine stimulation of cardiac afferents. However, with respect to reflex effects on gastric motility the baroreceptor stimulation elicits only a faint response while nicotine stimulation of cardiac receptors produces a profound reflex increase of gastric volume. This shows that the gastric relaxation cannot be a secondary phenomenon to the arterial hypotension as such as induced by heart receptor

stimulation. High threshold electrical stimulation (20 imp/s, 2 ms, 4 V) of the aortic nerve carrying fibres from the aortic arch, or of the carotid sinus nerve also produced marked reflex bradycardia and hypotension but had no significant effects on gastric motility Sometimes, however, a slight gastric relaxation could be seen when such stimulation characteristics were used that also chemoreceptor afferents in these nerves could be expected to become activated In one experiment it was observed that low threshold electrical stimulation of the aortic nerve (100 imp/s, 0 02 ms, 4 V) which reduced the blood pressure, had a clearcut excitatory effect on the stomach, this finding was not further investigated in this connection

Discussion

The present study has shown that electrical stimulation of vagal afferents from the heart or various types of cardiac receptor activation can induce considerable reflex relaxation of the stomach besides reflex circulatory changes This gastric relaxation could be so pronounced as to cause a 2—3 fold increase of gastric volume at a constant intragastric pressure This particular reflex response was shown to be specifically elicited from heart receptors and could not be ascribed to either any accidental direct excitation of efferent vagal fibres to the stomach or to secondary adjustments to the reflex circulatory responses Neither could the gastric reflex response be due to a generalized non specific excitation of the vagal motor neurons to the stomach Thus intense stimulation of arterial baroreceptor afferents that produced even more tensive reflex hypotension and bradycardia did not significantly affect the stomach a reflex fashion Only when the carotid sinus nerve was stimulated with such stimulus characteristics as to cause a reflex pressor response indicating a chemoreceptor fibre activation did a minor gastric relaxation occur This type of relaxation is however probably due to a reflex increase of the sympathetic outflow to the stomach

With regard to the location and functional characteristics of the cardiac receptors responsible for the reflex gastric relaxation, data from the present experiments strongly suggest that they are located mainly in the left ventricle Mechanical stimulation of the exposed epicardial surface of this ventricle or sudden rises of left ventricular pressure as produced by aortic occlusion causes a prompt and pronounced reflex gastric relaxation Pressure elevations in the right ventricle did not produce any clear gastric relaxation until the obstruction was released Then a prompt gastric relaxation occurred which was probably related to a sudden inrush of blood into the pulmonary system and left heart with a consequent mechanical distension of the left ventricle

Earlier experiment on reflex effects from the central circulation have shown that changes in left ventricular pressure can elicite marked depressor effects on the periferal circulation (Daly and Verney 1927 Aviado and Schmidt 1955) In contrast increased pressure in the right side of the heart causes only moderate reflex effects on the periferal circulation (Aviado *et al* 1951 Barer and Kottegoda 1958) Thus the left ventricle seems to be the most important part of the heart in evoking reflex circulatory reflexes

Local application of nicotine on the epicardial surface of the left ventricle also produced a marked reflex relaxation of the stomach suggesting that at least part of the receptors may be located to the epicardium or to outer myocardial layers It has earlier been described that nicotine intrapericardially elicits a marked depressor reflex (Sleight 1964, Bergel and Makin 1967) due to activation of C fibers in the left ventricular epicardium (Sleight and Widdicombe 1965)

The fact that the reflex responses to veratrum alkaloids administred i v were eliminated by application of a local anesthetic in the pericardial cavity also suggests that the receptors are located in the heart. Veratrum alkaloids have been showed to activate left ventricular receptors (Dawes 1947) with non myelinated afferents (Öberg and Thoren to be published)

Stimulation of the cardiac vagal afferents elicits marked depressor reflexes (Neil and Zotterman 1950 Öberg and White 1970 a) but first when the non myelinated fiber group is activated (Öberg and Thoren to be published) From the present experiments where the right cardiac nerve was electrically stimulated it was evident that also the reflex gastric relaxations were elicited by non myelinated afferent fibers

Histologically 75 % of the afferent fibers in the cardiac nerve are non myelinated (Agostini 1957) and at least part of them emanate from ventricular receptors (Jarisch and Zotterman 1948 Sleight and Widdicombe 1965 Öberg and Thoren to be published) It can therefore be concluded that the reflex gastric relaxation described in the present study emanates fom ventricular receptors signalling in non myelinated afferent fibers The receptors are probably mechanoreceptors responding to distension of the left ventricle or to dilatation of part of its wall in case its nutrition is seriously hampered so that bulging occurs during systole

Reflex circulatory effects from cardiac receptors during coronary occlusion have earlier been described (Kolatat *et al* 1967 Costantin 1963) and it was recently observed that left ventricular receptors with non myelinated afferents are activated during obstruction of a coronary artery (Thoren to be published) During coronary occlusion even sympathetic afferents probably nociceptive from the heart can be activated (Brown and Malliani 1971) but it is not known so far whether such afferents too may elicit reflex effects on gastric motility

The ventricular receptors are also activated by a rapid severe hemorrhage probably by squeezing and tearing of the myocardial fibers when the heart muscle is contracting vigorously around an almost emptied chamber as suggested by Pearce and Henry (1955) and recently experimentally illustrated (Öberg and Thoren 1970) In the present experiments gastric relaxation occurred during rapid bleeding regularly associated with reflex vagal bradycardia. Whether this gastric response to some extent was due to reflex effect from heart receptors was not definitely established in the present study

From the stimulations of the ventricular afferent C fibres it was evident that even such low frequencies as 1—2 imp/s were capable of producing significant reflex circulatory and gastric responses These two types of reflex responses to electrical

stimulation differed, however, in some respects. Thus, maximal circulatory adjustments were obtained already at frequencies of about 15 imp/s, while maximal gastric responses were obtained first at frequencies around 40—60 imp/s.

Preliminary experiments (Abrahamsson and Thorén, to be published) indicate that the efferent pathway in the described reflex effects on the stomach is mainly constituted by the vagal relaxatory fibers earlier described by Martinson (1965). This was supported by the findings that the promptly elicited reflex gastric relaxations persisted after administration of cholinergic and adrenergic blockers. These specific relaxatory fibers to the corpus fundus region of the stomach are known to be reflexly activated by distension of the esophagus or by swallowing (Abrahamsson and Jansson 1969). The present study shows another mechanism by means of which this fibre system can be reflexly activated, namely from the cardiac receptors, situated mainly in the left ventricle and connected to vagal C fibres. The functional significance of such a reflex mechanism is difficult to evaluate. It is possible, however, that the gastric relaxation constitutes an early component in a complex vomiting reflex pattern. It has been observed (Cannon 1911, p. 57) that in the initial phase of vomiting there is a marked loss of tone in the cranial part of the stomach. If this connection between ventricular receptors, the bulbar vomiting centre and vagal fibres to the stomach exists some well recognized clinical observations can be explained. It is known that a coronary infarction is often associated with nausea and sometimes gastrointestinal disturbances. Rapid bleeding is often capable to activate the same type of ventricular receptors, eliciting a reflex cardiovascular pattern closely similar to the vaso-vagal syncope reaction in man. This reaction in man is often accompanied by nausea and vomiting.

Other procedures known to activate such ventricular receptors are also combined with gastrointestinal disturbances. The veratrum alkaloids are for instance known to produce nausea and vomiting; their emetic effect was by Borison and Fairbanks (1952) thought to be triggered by drug effect on the nodose ganglion. The present study, however, suggests that ventricular receptors are likely to be of importance for veratrum induced gastrointestinal disturbances. It is also interesting that digitalis in large doses can produce a Bezold Jarish reflex (Melville 1952) and activate non myelinated afferents from the left ventricle (Öberg and Thorén, to be published).

Further intrapericardial injections of nicotine, which is known to stimulate cardiac receptors and according to the present study produces reflex gastric relaxation, did not precipitate vomiting when injected intrapericardially in conscious dogs (Sleight 1964) but besides a reflex blood pressure fall sometimes both hyperpnoea and salivation which also are components of the vomiting act. The failure to produce definite vomiting might be a question of the amount of nicotine administered. As shown in the present study a maximal relaxatory effect on the stomach necessitates a very powerful receptor activation with impulse discharge rates of 40—60 imp/sec while maximal circulatory effects are produced by far more moderate levels of receptor activities. It is possible that the reflex gastrointestinal effects are displayed only in some pathophysiological situations or during more extreme episodes in an

otherwise healthy organism *e g* when the left ventricle physically becomes either nearly emptied or overloaded

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examined before and after registration Suitable respiration is also important for effective elimination of the tracer and hence prevention of recirculation After the registration blood was collected in capillary tubes after a small incision in the splenic vein The tubes were sealed at one end and centrifugated at $9000 \times g$ for 5 min to determine the hematocrit Finally the spleen was removed and weighed

Calculation of splenic blood flow

The theoretical principles for blood flow measurements using inert gases have been presented by Kety (1960) and many others It can be shown that the logarithm of the concentration of
oncentration of the tracer
omogeneously perfused, in
is often a composite one
ie calculation of the blood
equilibrium of the tracer
tissue only *via* the efferent
blood

Thanks to the properties of xenon two of these requirements are immediately fulfilled Because the substance is more or less completely eliminated from the blood in one passage through the lung (Farhi 1967) the recirculation is very low (Sejrsen and Tonnesen 1968 Grandchamp *et al* 1971) An instant diffusion equilibrium of xenon between blood and tissue is also claimed (Ladefoged 1966)

In case of homogeneous perfusion the blood flow can be calculated from the formula

$$F = k\lambda \tag{1}$$

where F is the blood flow in ml/g min, k is the clearance constant $\left(\frac{\ln 2}{T\frac{1}{2}}\right.$, where $\ln 2 = 0\,693$ and $T\frac{1}{2}$ is the half life in min of the disappearance rate of the tracer substance) and λ is the partition coefficient which expresses the relationship between the concentration of the gas in the tissue and the blood at equilibrium The value of λ changes with the hematocrit according to Andersen and Ladefoged (1965) it can be corrected for the hematocrit (Hct) by means of the formula

$$\lambda Hct_x = \lambda Hct_{50} \frac{1\,69}{1\,05 + 0\,013\ Hct_x} \tag{2}$$

In the present investigation $\lambda_{Hct_{50}}$ was taken as 0 7 the approximate value obtained for several tissues in a number of investigations, some including the spleen (Conn 1961 Andersen and Ladefoged 1967 Rosendorff and Luff 1970) From (1) and (2) we have

$$F = \frac{0\,693 \times 1\,69\,\lambda_{Hct_{50}}}{T\frac{1}{2}\,(1\,05 + 0\,013\ Hct_x)} \tag{3}$$

When the wash-out curve has more than one component the mean blood flow can be calculated as the weighted arithmetic mean (Ladefoged 1966, Grandchamp *et al* 1971)

$$F = \frac{\sum_{i=1}^{n} k_i Q_{i\,0}}{\sum_{i=1}^{n} Q_{i\,0}/\lambda_i} \tag{4}$$

where n is the number of components and $Q_{i\,0}$ the initial amount of tracer substance The formula assumes a uniform initial distribution of the tracer substance in all perfusion areas this is probably the case after local application of the tracer but not so after intra arterial injection (Ladefoged 1966) When n = 2 this expression combined with (3) gives

$$F = \left(\frac{R_1}{(T\frac{1}{2})_1} + \frac{R_2}{(T\frac{1}{2})_2}\right)\frac{0\,693 \times 1\,69\,\lambda_{Hct_{50}}}{1\,05 + 0\,013\ Hct_x} \tag{5}$$

where $R_1 = \frac{Q_{1\,0}}{Q_{1\,0}+Q_{2\,0}}$ and $R_2 = \frac{Q_{2\,0}}{Q_{1\,0}+Q_{2\,0}}$ • In this formula λ is assumed to be the same for all perfusion areas

The ^{133}Xe wash-out curves were resolved into one rapid and one slow component (Fig 1) The curves from various animals often differed in appearance particularly as regards the slope and length of the initial part, this is probably because of different initial amounts of

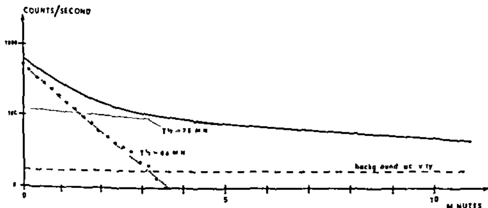


Fig 1 Xenon 133 wash-out curve from one of the animals in this investigation The semi logarithmical curve was recorded automatically It represents the sum of two components with different half life values (T 1/2) The final linear portion of the curve (representing the slow component) was extrapolated to zero time and subtracted from the curve to obtain the fast component (skippled line)

radioactivity in the two components The rapid component of the wash-out curve was obtained graphically by substracting the slow component which was extrapolated from the last linear portion of the curve The half lives of the two components were calculated from the curves The ratio of the radioactivity in the two components to the total radioactivity at the start of the registration (corresponding to R_1 and R_2 in (5)) was determined from each curve after subtracting the background The total splenic blood flow was obtained as the product of the mean flow, given by (5), and the weight of the spleen The specific blood flow was also calculated for each of the two components separately

To find the extent to which differences in the shape of the curves were due to methodological variations rather than to differences in blood flow, replicate measurements were performed in 16 guinea pigs The second injection was performed after the activity of the first injection had fallen to the background level and at least 5 mm from the first injection site In order to study the possible role of clearance mechanisms other than blood flow—for example diffusion of the xenon throughout the body or into the air—measurements were also made on some guinea pigs sacrificed by an overdose of Nembutal and in a few cases on the removed spleen

The rate at which lymphocytes were released from the spleen was calculated from the blood flow and the splenic veno-arterial difference in number of lymphocytes The standard deviation of the mean product was obtained from the formula

$$S_{x\bar{y}} = \pm \sqrt{x^2(S_y)^2 + y^2(S_{\bar{x}})^2} \qquad (6)$$

where x = mean blood flow
y = mean veno- arterial difference
$S_{\bar{x}}$ = standard deviation of $\bar{x}$
S_y = standard deviation of y

Tests of significance were performed with the Student s *t* test

Results

Splenic blood flow

The results of the blood flow measurements are presented in Table I In the normal animals the flow was greatest in the smallest animals (0 85 ml/g min) and it decreased steadily with body weight The value in the animals weighing 500 g (0 66

TABLE I Calculation of splenic blood flow in normal guinea pigs of 3 weight classes and in guinea pigs immunized with SRBC or pertussis vaccine The values were obtained from the ^{133}Xe clearance curves after resolution into two linear components (Fig 1) $T^1/_2$ The half life of the rate of disappearance of ^{133}Xe in the 2 components R_1, R_2 The ratio of radioactivity initially in the fast and slow component, respectively, to the total activity Means ± standard deviation of the mean

	Weight of spleen mg	Fast component			Slow component			Blood flow	
		$(T^1/_2)_1$	R_1	Blood flow ml/g min	$(T^1/_2)_2$	R_2	Blood flow ml/g min	ml/g min	µl/min
Normal groups									
150 g	229 *±25*	0 496 *±0 034*	0 776 *±0 042*	1 077 *±0 067*	8 12 *±0 68*	0 224 *±0 042*	0 0682 *±0 0069*	0 852 *±0 064*	194 *±24*
300 g	452 *±32*	0 565 *±0 045*	0 714 *±0 050*	0 957 *±0 076*	6 46 *±0 79*	0 286 *±0 050*	0 0869 *±0 0077*	0 727 *±0 085*	333 *±48*
500 g	968 *±97*	0 514 *±0 019*	0 633 *±0 065*	0 987 *±0 033*	7 01 *±1 04*	0 367 *±0 065*	0 0830 *±0 0092*	0 655 *±0 064*	638 *±90*
SRBC									
Immunized	412 *±22*	0 523 *±0 047*	0 749 *±0 062*	1 095 *±0 104*	7 43 *±0 60*	0 251 *±0 062*	0 0761 *±0 0069*	0 854 *±0 114*	362 *±60*
Controls	365 *±15*	0 502 *±0 078*	0 760 *±0 045*	1 211 *±0 128*	8 06 *±1 44*	0 240 *±0 045*	0 0784 *±0 0088*	0 973 *±0 126*	360 *±53*
Pertussis									
Immunized	409 *±28*	0 501 *±0 031*	0 743 *±0 034*	1 098 *±0 066*	6 81 *±0 37*	0 257 *±0 034*	0 0807 *±0 0056*	0 852 *±0 073*	349 *±39*
Controls	428 *±29*	0 611 *±0 040*	0 651 *±0 040*	0 905 *±0 060*	6 93 *±0 34*	0 349 *±0 040*	0 0786 *±0 0032*	0 625 *±0 050*	266 *±26*

ml/g min was significantly lower ($p < 0\ 05$) This gradual reduction in blood flow with weight seems to be due to a change in the relative magnitude of the 2 flow components (R_1 and R_2 in Table I) rather than to an alteration of the flow within each component

After immunization with pertussis vaccine the half life of the rapid component was shorter than in the corresponding controls ($p < 0\ 05$) and the amount of radioactivity initially trapped in the rapid component was greater, reflecting an increase in blood flow per g of spleen tissue ($p < 0\ 05$ Immunization with SRBC had no definite effect on splenic blood flow

The hematocrit ranged from 33 to 49 mean 40 4)

Control experiments on blood flow measurements

From the replicate measurements on the same animal it was found that variations in the shape of the clearance curves were clearly more related to differences between individual spleens than to any unknown methodological factors The statistical significance of this observation was tested by comparing the intra- and inter subject

TABLE II Output of lymphocytes from the spleen (cells per min × 10⁻⁶) The values were obtained as the product of the mean blood flow (µl per min) and the mean difference between splenic efferent and afferent blood (cells per µl) The standard deviation of the product was obtained from the formula

$$S_{\bar{x}y} = \pm\sqrt{x^2 (S_y)^2 + y^2 (S_x)^2}$$

	Output
Normal groups	
150 g	0 81 ± *0 20*
300 g	3 90 ± *0 99*
500 g	7 66 ± *2 47*
SRBC	
Immunized	2 35 ± *0 63*
Controls	1 53 ± *0 66*
Pertussis	
Immunized	2 10 ± *0 67*
Controls	0 33 ± *0 15*

variances The variance in the blood flow values due to the inter subject differences was nearly 6 times greater, this difference between the variances is significant ($p < 0.01$), and indicates that the experimental errors were sufficiently small for obtaining reproducible results For none of the parameters was the difference between the first and second measurements different from zero, the values yielded by the second measurement were thus not influenced by the first

For the animals killed by an overdose of Nembutal the clearance curve after injection of isotope into the spleen was practically horizontal indicating no elimination of the isotope The same is true for the isolated spleens This suggests that it is only the blood flow that is essentially responsible for the clearance of isotope

Splenic release of lymphocytes

From the blood flow and earlier determinations of the difference in lymphocyte content of splenic efferent and afferent blood (Ernstrom and Sandberg 1968 Sandberg 1970 a b) the total release of lymphocytes formed in the spleen was obtained (Table II) Since there is also a migration of lymphocytes into the spleen from other lymphoid organs (Linna and Stillstrom 1966 Brahim and Osmond 1970) these values are minima An increasing migration of lymphocytes into the blood with age was demonstrated (Table II) The fact that 12 days after immunization with pertussis antigen there was an increase in the splenic blood flow and the veno-arterial difference in the number of lymphocytes (and also in granulocytes and monocytes) means that at this time a greater number of cells leaves the spleen *via* the efferent blood vessels due to the pertussis injection ($p \simeq 0.001$) At 6 days after immunization with SRBC there was an increased release of splenic lymphocytes which however, is not statistically significant (Table II)

Discussion

For a homogeneously perfused tissue the wash out curve of an inert gas (such as xenon or krypton) should be monoexponential, being represented by a straight line when plotted semilogarithmically, but this is seldom the case, and was not so in the present study A two component curve is found in skeletal muscle, liver and skin while in the kidney three or four components have been reported It is noteworthy that Blendis *et al* (1970) obtained ^{133}Xe wash out curves for the dog spleen which all indicated a single exponential clearance rate Using the same technique and experimental animal, Zetterstrom (1971) instead found evidence of a two component course of the clearance However, he had noted that in cases of experimentally induced decrease of the splenic blood flow, a single component occurred In this context it is of interest to note that ^{133}Xe clearance curves for the human quadriceps *muscle at rest were monoexponential while those recorded during exercise were* composite (Grimby, Haggendal and Saltin 1967)

It has been proposed that the different components of the clearance curves correspond to anatomical zones (Thorburn *et al* 1963, Thorburn, Casey and Molyneux 1966, Sejrsen 1969 Grandchamp *et al* 1971) Other suggested mechanisms include counter current exchange (Sejrsen and Tonnesen 1968 Lewis 1970), recirculation of the tracer substance (Ladefoged 1966) and activity originating outside the measured organ (Darle 1970) As for the physiological significance of the two components in ^{133}Xe wash-out curves for the spleen in the present investigation, only tentative explanations can be offered pending further studies One possibility that mes to mind is that the fast and slow components represent the red and white lenic pulp respectively

The ^{133}Xe may be introduced into the splenic tissue either by an intra arterial injection or as in the present case by injection directly into the tissue The former technique was tried and found to be less satisfactory in these small animals the splenic artery cannot be cannulated without considerable manipulation which invariably results in impairement of the blood flow due to constriction of the artery The injection directly into the tissue also causes some trauma which may affect the local blood flow and the clearance of the xenon but the amount injected was small and as is seen from the replicate measurements the first injection did not modify the blood flow in the spleen as a whole

There would seem to have been no investigation concerned with the quantification of splenic blood flow in the guinea pig but for the dog measurements have given values of 0 1 (Ottis Davis and Green 1957) 0 58 (Burton Opitz 1909) and 1 28 ml/g min (Koyama 1967) From comparison of the results of ^{133}Xe measurements with those obtained with an electromagnetic flowmeter Blendis *et al* (1970) concluded that the ^{133}Xe technique of measuring organ blood flow is valid for the spleen Their values in respect of the dog spleen were all below 0 4 ml/g min Flow measurements with ^{133}Xe were performed on the dog spleen also by Zetterström (1971), who measured flows between 0 3 and 1 0 ml/g min in the exteriorized spleen during slight Nembutal anesthesia The reason for the lower blood flow in

the experiments of Blendis *et al* (1970) could be due to manipulation with electromagnetic flowmeters or a greater depth of narcosis This suspicion is strengthened by the fact that their wash-out curves could be resolved into one component only (see above) Using the xenon technique on the human spleen, Williams *et al* (1966) measured a flow of 0 96 ml/g min

Combination of blood flow values with earlier determinations of the splenic veno-arterial difference in number of lymphocytes (Ernström and Sandberg 1968, Sandberg 1970 a b) permitted the quantification of the release of lymphocytes from the spleen. In the normal guinea-pigs the largest number of emigrating cells was recorded in the heaviest animals In 24 h 10^9 more lymphocytes left the spleen than entered it, provided that the measured veno-arterial differences in lymphocyte number are continuous for this period of time this figure corresponds to the approximate daily production of lymphocytes in the bone marrow of a 400 g guinea pig (W H O 1970) This would indicate that the role of the spleen in lymphocyte production is greater than is generally acknowledged

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Postnatal Development of the Mechanical Response of the Isolated Rat Vas Deferens to Nerve Stimulation

By

GORAN SWEDIN

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Abstract

SWEDIN, G *Postnatal development of the mechanical response of the isolated rat vas deferens to nerve stimulation* Acta physiol scand 1972 *84* 217—223

Isolated vas deferen [illegible] 22 d [illegible] are stimulated transmurally in [illegible] in prepara- [illegible] (NA) and [illegible] parations of all ages responded readily to low concentrations of exogenous NA (0 2—5 0 μg/ml) while acetylcholine (ACh) had to be added in much higher concentrations (10—100 μg/ml) to give equal direct responses

α receptor blockers (phentolamine or phenoxybenzamine, 0 2—2 0 μg/ml) totally inhibited the contractions of preparations from animals up to about 10 days of age but thereafter the effect was changed into potentiation of the responses Atropine (0 5—2 0 μg/ml) generally caused a slight decrease of contractions of preparations up to about 15 days of age, but at later stages [illegible]

[illegible] liberated at these early ages

In a previous investigation (Owman, Sjöberg and Swedin 1971) the pre and postnatal development of the adrenergic innervation of different peripheral organs of the rat was studied histochemically with special reference to the different system of "long" and "short" adrenergic neurons It was shown that, in most organs, a varicose terminal adrenergic network was not established until, or immediately after, birth

The present study was performed in order to establish the functional development of the adrenergic innervation of the rat vas deferens as indicated by the appearance of a mechanical response of the longitudinal smooth muscle layer to transmural nerve stimulation

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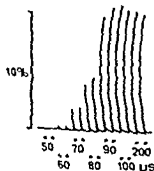




Fig 3

Fig 4

Fig 3 Isolated rat vas deferens (*13 days* total length of preparation 18 mm) field stimulated for 5 s at 1 min interval with 20 V, 10 imp/s of different duration (µs) as indicated on the abscissa. In Fig 3—7 the amplitude of the contractions are compared (%) to the total length of the preparations

Fig 4 Isolated rat vas deferens preparation (*3 days* length 15 mm) field stimulated for 5 s at 1 min interval with 20 V, 20 imp/s 0 2 or 10 ms duration as indicated in the fig PBA phenoxybenzamine 0 5 µg/ml

Results

The threshold duration of the stimulus for evoking a nerve induced contraction of the organ was generally distinct between 0 05 and 0 06 ms with maximum amplitude obtained with a pulse length between 0 1 and 0 6 ms (Fig 3) This nerve-induced contraction, which was heavily depressed or abolished by *guanethidine* (5 µg/ml), is readily distinguishable from the direct smooth muscle response, which after adrenergic receptor or neuron blockade began to develop at about 2 ms duration with maximum between 5 and 10 ms

In Fig 3—7 the mechanical responses of the rat vas deferens to field stimulation or exogenous NA and ACh are related to the total length of the preparations As can be seen there is a gradual increase in the percentual shortening of the organs on nerve or smooth muscle stimulation from less than 0 1 % at 3 days (20 imp/s, Fig 4) up to about 10 % at 15 days (10 imp/s Fig 3 and 7 C)

The first nerve induced responses were obtained in preparations from 3 days old animals (Fig 4) The responses were blocked by *phentolamine* or *phenoxybenzamine* (0.5 µg/ml, Fig 4) whereafter only direct smooth muscle contractions were ob-

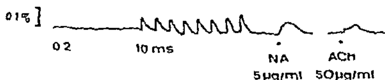


Fig 5 Isolated rat vas deferens preparation (*2 days* length 10 mm), field stimulated for 5 s at 1 min interval with 20 V, 20 imp/s 0 2 or 10 ms duration. Direct responses to NA and ACh.

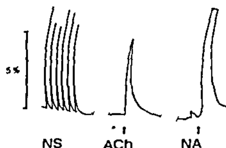


Fig 6 Isolated rat vas deferens preparation (10 ... al) ... A

tained One day earlier (2 days), only direct muscle contractions and responses to exogenous NA and ACh were observed (Fig 5)

The effect of exogenous *NA* and *ACh* are further illustrated in Fig 6 (10 days) Most preparations responded readily to NA concentrations of 0 2—1 0 μg/ml but even 20 ng/ml evoked a direct response in some preparations, in the youngest preparations somewhat higher concentrations were usually needed (*cf* Fig 5) ACh had to be added in much higher concentrations (10—100 μg/ml) to give contractions of equal amplitude These direct effects of NA and ACh were totally abolished by phentolamine and atropine (0 5—1 0 μg/ml) respectively

The effect of adrenergic and cholinergic receptor blockers on the responses of the vasa deferentia of different ages to nerve stimulation are shown in Fig 7 A B and C At all ages between 3 and 10 days the *adrenergic a blocking agents* (phentolamine or phenoxybenzamine 0 2—2 0 μg/ml) caused total inhibition of the nerve-induced contractions together with a slight reduction of the tone of the organs (Fig 4 7 A B) Between the 11th and 15th days the effect of these drugs were changed to none or a potentiation of the responses to nerve stimulation (Fig 7 C) Also the effect of *atropine* (0 5—2 0 μg/ml) changed during the postnatal development In the earlier stages (3—15 days) there was generally a slight or moderate (10—40 %) decrease of the contractions (Fig 7 B and C) which were not further influenced by increasing the contraction of atropine up to 5 μg/ml In preparations from older animals atropine was without effect

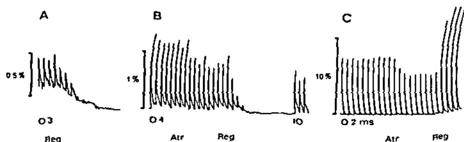


Fig 7 Isolated field stimulated ... Duration (ms) of the stimuli indicated in ... is 0 3 ms Reg phentolamine 2 μg/ml B ... ms duration Atr atropine 2 μg/ml Re... m 20 V 10 imp/s 0 2 ms Atr atropine 1 μg/ml Reg phentolamine 1 μg/ml

Discussion

In the present study on the postnatal development of the motor innervation of the rat vas deferens a superfusion bath was used Since no gas bubbles interfered with the mechanical recording system the sensitivity of the system could be increased so as to permit registration of contractions with an amplitude of less than 0 01 mm (*cf* Fig 5)

The first responses to transmural nerve stimulation were obtained in preparations from 3 days old animals Response to exogenous NA and ACh and direct contractions of the smooth muscle cells were observed one day earlier Preparations of all ages responded readily to low concentrations of exogenus NA while ACh had to be added in 20—100 times higher concentrations to give equal direct responses α receptor blockers totally inhibited the nerve induced contractions of preparations from animals up to about 10 days of age but thereafter the effect was changed into none or a potentiation Atropine generally caused a slight decrease of contractions of preparations up to about 15 days of age but at later stages it was without effect

Recently the perinatal outgrowth of the adrenergic neurons to different peripheral organs of the rat has been studied with the Falck Hillarp histofluorescence method (de Champlain *et al* 1970, Owman Sjoberg and Swedin 1971) The short adrenergic neurons innervating the internal genital organs appeared in the periphery of vas deferens during the first 2 days after birth and 2—3 days later they formed a net work radiating through the smooth muscle layers A dense ground plexus was first formed in the innermost parts from where it then rapidly extended in a concentric manner to occupy the entire thickness of the musculature 8—10 days after birth

The present investigation indicates a close correlation between morphological and functional development of the terminal adrenergic innervation of the rat vas deferens This is somewhat in contrast to the case with the mouse vas deferens where functional transmission as indicated by the registration of excitatory junction potentials does not occur until 18 days postpartum (Furness McLean and Burnstock 1970) when the noradrenergic nerves already are fully developed morphologically (Yamauchi and Burnstock 1969 b Furness McLean and Burnstock 1970)

There has been some confusing points with regard to the postganglionic adrenergic innervation of the vas deferens One is the repeatedly reported observation that adrenergic α receptor blockers do not inhibit but rather enhance the responses of the isolated vas deferens to nerve stimulation in concentrations (0 1—5 μg/ml) sufficient to abolish the responses to exogenous NA (Boyd Chang and Rand 1960 Kuriyama 1963 and others) 100—1000 times higher concentrations are generally needed to inhibit the nerve induced contractions

It is known that the nerve induced contractions of the pelvic viscera are difficult to block pharmacologically (*cf* Gruber 1933 Ursillo 1961) Since the action of the receptor blockers generally is competitive in nature and the nerve stimulation is apt to cause a high local concentration of transmitter in the junctional gap it seems reasonable that high exogenous concentrations of the drugs should be necessa

give sufficient concentration at the junctional receptors, whereas the receptors elsewhere on the cell membrane should be more easily reached from the extracellular space (Hotta 1969, *cf* Holman 1970) In analogy, exogenous NA should exert its main effect on the extra junctional receptors (Holman 1967, Hotta 1969), which fits well with the fact that this effect is readily blocked by moderate concentrations of *a* blockers (*cf* Bentley and Smith 1967)

The resistance of the nerve induced contractions of the adult vas deferens to *a* blockers could in part be due to the dense arrangement of the smooth muscle fibres in combination with unusually narrow neuromuscular junctions (Richardson 1962, *cf* Burnstock 1970) In the present study even low concentrations of *a* blockers were able totally to abolish the nerve induced contraction up to about 10 days of age Studies on the mouse vas deferens (Yamauchi and Burnstock 1969 a) has indicated a much greater extracellular space of the vas deferens from newborn and young animals suggesting more favourable diffusion properties in these young organs Another factor might be a smaller relative release of NA in the young preparations

The other topic of much controversy has been the participation of cholinergic mechanisms in the motor innervation of the vas deferens Morphological (Bell and McLean 1967 Burnstock and Robinson 1967 Robinson 1969) as well as pharmacological (Birmingham and Wilson 1963 Della Bella Benelli and Gandini 1964 Bhargava Kar and Parmar 1965 Birmingham 1966) and electrophysiological (Bell 1967) arguments have been presented strongly indicating the existence of an independent postganglionic cholinergic component in the guinea pig vas deferens The conflicting results regarding the atropine resistance of this innervation (*cf* Bell 1969) might be due to penetration difficulties in the adult tissue (*cf* Dale and Gaddum 1930 Ursillo 1961) as seems to be the case for the *a* blockers That the cholinergic component plays only a minor role in the rat vas deferens is indicated by the following facts 1 The sensitivity of the rat vas deferens to exogenous ACh is very low much lower than that of the guinea pig (Sjostrand 1961) 2 Atropine gives only a slight reduction of the responses also in the very young preparations 3 After adrenergic *a* receptor blockade the released amounts of ACh are insufficient for a registrable mechanical response of the vas deferens to transmural nerve stimulation Most probably the released ACh is functioning as potentiating agent for the released NA (Sjostrand 1961 Sjostrand and Swedin 1968)

That the major part of the early nerve induced contractions of the rat vas deferens reported in the present study in fact is mediated by adrenergic neurons liberating NA is indicated by the presence of receptors for NA which can be blocked by specific adrenergic *a* blockers which also totally abolish the nerve induced responses as do adrenergic neuron blockade by guanethidine

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Effect of Nerve Stimulation In Vitro on the Noradrenaline Content of the Rat Vas Deferens in the Presence of Inhibitors of Noradrenaline Uptake and Synthesis

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Abstract

SWEDIN G *Effect of nerve stimulation in vitro on the noradrenaline content of the rat vas deferens in the presence of inhibitors of noradrenaline uptake and synthesis* Acta physiol scand 1972 *84* 224—230

Isolated rat vas deferens preparations were intermittently field stimulated (30 s every min) for 2 or 4 h at 7 or 25 imp/s At the low stimulation frequency only a very small reduction (7 %) of the endogenous noradrenaline (NA) stores was seen during 4 h stimulation while it was about 15 % after 2 h and 37 % after 4 h at 25 imp/s Addition of drugs known to inhibit NA synthesis (α methyl *p* tyrosine α MPT 4×10^{-4} M) or uptake (cocaine 10^{-5} M) did not apparently influence neither the spontaneous nor the nerve induced reduction of the endogenous NA stores The present results seem to support the hypothesis that the functional transmitter pool of the short adrenergic neurons of the vas deferens is very small and that the residual NA reuptake and synthesis after pharmacological blockade together with minute refilling from the large storage pools are able to maintain transmitter homeostasis without significant changes of the total endogenous NA content of the vas deferens

In a previous study (Swedin 1970) an attempt was made to measure the *in vivo* turnover of NA in the short adrenergic neurons innervating the vas deferens of the rat α methyl *para* tyrosine (α MPT) was given to rats to block the synthesis of NA (Spector, Sjoerdsma and Udenfriend 1965) which resulted in an exponential decline in the NA content of the heart and submaxillary gland while that of the vas deferens and seminal vesicle remained quite unaltered during at least 16 h

This lack of effect of inhibiting the NA synthesis might be due to absence of nerve impulse flow in the short adrenergic neurons *in vivo* Another explanation could be that these neurons function with a very efficient reuptake and reuse of liberated NA The present study was undertaken in order to evaluate these possibilities by measuring the effect of nerve stimulation *in vitro* on the NA content of the rat vas deferens in the presence of inhibitors of NA synthesis and reuptake

Material and methods

98 male Sprague Dawley rats (b w 370—400 g) were used. The rats were killed by a blow on the head and the vasa deferentia were d ssected out and mounted in two 5 ml organ baths one of wh ch had two parallel platinum electrodes (8 mm apart) in the wall The NA content of

timulated control or drug concentration between the d Harrison 1970 Swed n (B rmingham and Wilson

ige 1 ms duration and a frequency of 7 or de solution (37° C aerated with 6 5 % CO_2 NaCl 0 8 % KCl 0 02 % $CaCl_2$ 0 0? % % and glucose 0 1 %

The mechanical responses of the preparations were recorded isoton cally (load 0 2—0 5 g) on a Grass Polygraph

The following drugs were used dl-α methyl *para* tyrosine methylester hydrochloride (H 44/68 Hassle) cocaine hydrochloride desmethylimipramine (DMI Pertofrin® Geigy) and phenoxybenzam ne hydrochloride (PBA D benzyline® Smith Kl ne & French) α MPT was added 10 min before the stimulation period the other drugs at the beginning of stimu lation

Extract on and NA determination After the experimental periods the vasa deferentia were we ghed and homogenized (single organs) by grind ng in 2 ml of icecold 0 4 N perchloric ac d NA was extracted on alumina as previously described (Sjostrand and Swedin 1968) and determined fluorimetrically according to Chang (1964) The NA is expressed as μg free base

d water washings from the alumina the sum of endogenous tyrosine and

Results

The intermittent nerve stimulation (30 s every min) resulted in a mechanical response which usually after an initial period of moderate decline remained quite unaltered for several hours α MPT did not influence the mechanical response of the vas deferens to nerve stimulation while cocaine in the concentration used always caused a marked potentiation of the contractions at both 7 and 25 imp/s

Table I summarizes the effect of nerve stimulation *in vitro* on the endogenous NA content of the rat vas deferens in the presence of α MPT (4×10^{-4} M) or cocaine (10^{-5} M) or a combination of both drugs The spontaneous NA reduction on Tyrode incubation (37° C) only was for 2 h about 19 % ($0.05 > p > 0.01$) and for 4 h about 25 % ($0.01 > p > 0.001$) The intermittent nerve stimulation induced no decrease of the endogenous NA levels *per se* during the first 2 h at the lower stimulation frequency (7 imp/s while there was about 15 % loss in 2 h at 25 imp/s After 4 h of stimulation the reduction was about 7 % and 37 % at 7 and 25 imp/s respectively

Addition of α MPT or cocaine or both drugs did not in the present concentra tions influence the spontaneous loss of NA of the incubated but unstimulated organs when compared to the unstimulated organs in the drugfree Tyrode ($p > 0.05$) Neither do these drugs seem to increase the nerve induced decrease of endogenous NA Endogenous contents of tyrosine was found to be 36 ± 2 μg/g in vasa deferentia incubated for 2 h in Tyrode (n = 18) while those incubated for 2 h with α MPT 4×10^{-4} M contained 249 ± 13 μg/g (tyrosine + α MPT n = 16)

Some experiments were also performed with DMI and PBA (Table II)

TABLE I Effect of nerve stimulation (30 s every min) *in vitro* and of addition of α MPT and cocaine when not otherwise indicated α MPT was added 10 min before the stimulation period (t test) to nonincubated controls, all unstimulated drug incubated organs compared **0 01 > p > 0 001, *** p < 0 001

Time (h)	Stimul freq (imp/s)	No drug Unstim	Stim	Mean % NA remaining	α MPT 4x10⁻⁴M Unstim	Stim	Mean % NA remaining
0	—	11 96±0 81 (n = 10)	—	—	—	—	—
2	7	8 78±0 41	8 81±0 30	100	8 38±0 90	8 46±0 89	101
	25	10 62±0 55	9 06±0 29	85	9 79±0 63	8 10±0 28	83
		9 70±0 47* (n = 8)			9 09±0 57* (n = 8)		
4	7	8 65±0 57	8 06±0 52	93	9 95±0 66	8 97±0 93	90
	25	9 33±0 56	5 89±0 16	63	7 86±0 44	5 43±0 72	69
		8 99±0 39** (n = 8)			8 90±0 54* (n = 8)		

to obtain a greater reduction of the endogenous NA than that caused by the nerve stimulation itself very high concentrations of the drugs had to be used (PBA 10^{-4} M DMI 10^{-5} M) The mechanical responses of the organs were totally abolished by both drugs in these concentrations Most of the observed reduction of the NA content is due to a direct effect of the drug while the nerve stimulation plays only a minor role Thus if the significant (0 01 > p > 0 001) direct effect of PBA (10^{-4} M) on the NA content of the unstimulated organs is compensated for the further decrease in the stimulated organs is just as great, about 15 %, as that obtained in the control stimulated preparations without PBA (*cf* Table I)

Discussion

The present study has shown that the endogenous stores of NA of the rat vas deferens are markedly resistant to nerve stimulation and that this resistance persists also in the presence of drugs known to inhibit uptake (cocaine) and synthesis (α MPT) of NA

It is well known that a moderate nerve impulse flow in adrenergic nerves does not normally influence the endogenous content of NA in different organs (Luco and Goñi 1948 Euler and Hellner Bjorkman 1955, and others) A partial depletion of transmitter on nerve stimulation has however been reported for *e g* the spleen (Dearnaley and Geffen 1966 a b) skeletal muscle (Kernell and Sedvall 1964) and submaxillary gland (Fredholm and Sedvall 1966) On direct comparison the NA stores of vas deferens seem to be more resistant to nerve stimulation than those of the spleen (Blakeley, Dearnaley and Harrison 1968 1970) and very intense and prolonged stimulation has been reported necessary for a measurable reduction of the transmitter stores of the vas deferens (Chang and Chang 1965 Roth Stjarne and Euler 1967, Palaic and Panisset 1969) To reduce the risk of unspecific fatigue

on the endogenous NA content of the rat vas deferens Mean ± s e m , n = no of expts n = 4 cocaine at the beginning of the stimulation Mean of all unstimulated controls at 2 and 4 h compared to unstimulated controls from 2 and 4 h respectively ° $p > 0.05$ * $0.05 > p > 0.01$,

Cocaine 10^{-4}M		Mean % NA remaining	α MPT 4×10^{-4}M + Cocaine 10^{-4}M		Mean % NA remaining
Unstim	Stim		Unstim	Stim	
—	—	—	—	—	—
9.23±0.35	8.92±0.70	97	9.33±0.90	8.75±0.72	94
9.27±0.65	8.58±0.86	92	9.00±0.92	7.64±0.57	85
9.25±0.34° (n = 8)			9.16±0.60° (n = 8)		
—	—	—	—	—	—
—	—	—	—	—	—

TABLE II Effect of nerve stimulation (25 imp/s 30 s every min for 2 h) *in vitro* on the endogenous NA content of the rat vas deferens in the presence of DMI or PBA added in the given concentrations at the beginning of the stimulation period Unstimulated drug incubated preparations compared (t test) to unstimulated controls ° $p > 0.05$ ** $0.01 > p > 0.001$ *** $p < 0.001$

	Stimulated	Unstimulated	Mean % NA remaining
No drug	—	9.73±0.34 (n = 7)	—
DMI 10^{-6} M	8.48±0.40 (n = 4)	9.17±0.43° (n = 4)	92
10^{-4} M	0.77±0.22 (n = 4)	2.15±0.31*** (n = 4)	—
PBA 10^{-4} M	6.74±0.25 (n = 8)	7.93±0.33** (n = 8)	85

phenomenon of the nerve terminals as a result of continuous high frequency stimulation intermittent (30 s every min) stimulation generally for 2 or 4 h was used in the present study Furthermore it has been shown (Weiner and Rabadjija 1968) that nerve stimulation for 30 s every min is about twice as effective as continuous nerve stimulation in increasing the NA synthesis of the guinea pig vas deferens

The effectiveness of α MPT as an inhibitor of the NA synthesis has been clearly demonstrated both *in vivo* and *in vitro* (Udenfriend Zaltzman Nirenberg and Nagatsu 1965 Udenfriend *et al* 1966)

The *in vitro* concentration of α MPT in the present study was determined so as to secure sufficient organ concentration of the drug and a molar ratio α MPT to endogenous tyrosine (T) of 6 1 was actually obtained With purified tyrosine hydroxylase a molar ratio of α MPT to T of 1 1 gives > 50 % inhibition of the enzyme *in vitro* (Udenfriend Zaltzman Nirenberg and Nagatsu 1965)

Cocaine was chosen as inhibitor of NA uptake in the present study for the following reasons 1 It is effective in preventing uptake of ^{3}H-NA in isolated guinea pig vas deferens (Bhagat and Zeidman 1970) and slices of rat vas deferens (Iversen 1968, Iversen and Langer 1969) 2 It has been shown to increase the NA output from the superfused vas deferens (Hughes 1970) without influencing the NA metabolism (Iversen and Langer 1969, Johnson, Thoa and Kopin 1971) 3 It has no α blocking activity (Osborne and Sigg 1960, Boullin, Costa and Brodie 1967) 4 Its effects on the mechanical and electrophysiological behaviour of the vas deferens have been studied This permits a choice of optimal *in vitro* concentration of the drug, above that which gives a definite potentiation of the mechanical response and summation of the junction potentials without any marked effect on the resting membrane potential (Bell 1967), but below that which causes spontaneous activity of the smooth muscle cells (Cliff 1968) or partial nerve blockade (Bell 1967, Greenberg and Long 1971)

DMI has also been shown to inhibit uptake of exogenous NA in slices of rat vas deferens (Haggendal and Hamberger 1967, Iversen 1968, Iversen and Langer 1969) but the direct effects of the drug on vas deferens is not known At those concentrations used in the present study it is not functioning only as an uptake blocker but also an α receptor blocker (Barnett, Symchowicz and Taber 1968), perhaps also with local anesthetic effects (Osborne and Sigg 1960) PBA is probably also acting by other mechanisms than blockade of NA uptake only (Hedqvist 1969 Farnebo and Hamberger 1970 Langer 1970, Wennmalm 1971), as indicated also by the direct effect of PBA 10^{-4} M without nerve stimulation in the present study

Despite these precautions in choice of drugs and concentrations, neither a blockade *of uptake nor synthesis of NA did increase the slight reduction of the endogenous* transmitter stores caused by the intermittent nerve stimulation *per se* The most probable reason for this seems to be that the short adrenergic neurons of the vas deferens normally are working with a very small pool of functional transmitter Several studies on the heavily NA depleted vas deferens from reserpine pretreated animals speak in favour of this assumption (*cf* Bell 1969) No pharmacological blockade of uptake or synthesis is 100 % effective, and probably the residual function together with minute refilling from the large store pools are able to maintain transmitter homeostasis without significant changes of the total endogenous NA content of the vas deferens

This study has been suported by the Medical Faculty Karolinska Institutet

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Subcellular Localization of the Prostaglandin System in the Rabbit Renal Papilla

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Abstract

Änggård, E, S O Bohman, J E Griffin III, C Larsson and A B Maunsbach *Subcellular localization of the prostaglandin system in the rabbit renal papilla* Acta physiol scand 1972 84 231—246

The occurrence of prostaglandins (PG), prostaglandin precursor acids prostaglandin synthetase and 15-hydroxy prostaglandin dehydrogenase was studied in subcellular fractions isolated from homogenates of rabbit renal papilla by sucrose density gradient centrifugation The investigated fractions were lipid droplets, supernatant microsomes and mitochondria as verified by electron microscopy Most of the prostaglandins was found to be prostaglandin E_2 and was present in the supernatant whereas the prostaglandin synthetase occurred most abundantly in the microsomal fraction Nearly all of the recovered prostaglandins were formed during the preparation of the subcellular fractions Arachidonic acid was the major esterified C_{20} acid and had the

...branes which sediment in the microsomal fraction and which may be derived from either cell membranes or membranes of the endoplasmic reticulum After their formation the prostaglandins are probably released into the cytoplasm of the cells rather than being concentrated within specific subcellular particles and may leave the papilla without undergoing metabolic inactivation

The original discovery that the renal medu a contains appr ble quantities of prostaglandin like material (Lee *et al* 1963 ha e een ex ended in several directions The major prostaglandin was ide d as pro aglandin E (Daniels *et al* 1967 Lee *et al* 1967) or closely related con po nd in biosynthesis of PGE from ^{3}H labelled arachidonic acid was demonstrated in rabbit renal medulla by Hamberg (1969) PGF was observed to be released in renal venous blood after ischemia (McGiff *et al* 1970 a, Edwards Strong and Hunt 1969), nerve stimulation (Dunham and Zimmerman 1970) angiotensin (McGiff *et al* 1970 b) and into renal lymph following injection of noradrenaline (Fujimoto and Lockett 1969)

Since prostaglandin E-compounds antagonize the actions of antidiuretic hormone (Orloff, Handler and Bergstrom 1965, Orloff and Grantham 1966, Johnston, Herzog and Lauler 1967 a, b) and have strong natriuretic effects (Herzog, Johnston and Lauler 1966, Johnston *et al* 1967 b) a physiological role has been suggested for PGE_2 as an intrarenal modulator of salt and water excretion

The interstitial cells of the renal medulla contain numerous lipid droplets and have been assigned secretory functions (Gloor and Neiditsch-Halff 1965 Bulger and Trump 1966, Osvaldo and Latta 1966, Nissen 1967) The lipid droplets accumulate during salt loading in salt-depleted rats (Nissen 1968 a) and during hydration in water-depleted rats (Nissen 1968 b) A role for these droplets in prostaglandin formation was suggested (Nissen 1968 c)

The present study was undertaken to provide evidence for the localization of substrates and enzymes involved in PG-synthesis and metabolism in morphologically defined fractions of the renal papilla with special reference to the lipid droplets of the interstitial cells

Methods and Materials

One or two male untreated rabbits weighing 2 0 3 5 kg were used for each experiment The animals were anesthetized by i.v. injection of sodium pentobarbital 75—100 mg/kg or urethane 1 5 g/kg Prostaglandin E_1 (PGE_1) and eicosa 5 8 11 14-tetraynoic acid were obtained from the Upjohn Company through the courtesy of Dr J E Pike ^3H labelled PGE_2 (3 5 Ci/mmol) was prepared as described previously (Änggård, Green and Samuelsson 1965) ^3H Labelled PGE_1 (30 Ci/mmol) was purchased from New England Nuclear Corporation Boston Mass USA Both compounds had a radiopurity of >97 %. Whenever necessary small amounts of radioactivity with a mobility corresponding to PGA_1 or PGA_2 respectively were removed by chromatography on silicic acid (Samuelsson 1964)

15 Hydroxy prostaglandin dehydrogenase 3 mU/mg of protein was purified as described previously (Änggård and Samuelsson 1969) ^3H eicosa 5 8 11,14 tetraenoic acid (1 22 Ci/mmol) was purchased from N E N Corp Boston Mass USA The radiopurity was found to be in excess of 96 5 % as checked by radiogas chromatography after treatment with diazomethane

Pyridine nucleotides reduced glutathione N-ethyl maleimide and bovine serum albumin were supplied by [illegible] acetic acid (disodium [illegible]

Preparation of [illegible] and ly dissected out [illegible] all the material [illegible] The tissue was homog[illegible] man and Maunsbach 1971)

The homogenate was divided into 2 parts one [illegible] or in some experiments immediately boiled (see biochemical [illegible] which was fractionated as shown in Fig 1 according to the [illegible] man and Maunsbach 1971) Each gradient was loaded with [illegible] Two gradients were used in most expe[illegible] part of the homogenate was used for [illegible] ation gradients were then put together T[illegible] operated with the SW 25 1 or SW 50 1 [illegible]

The distribution of bands in the [illegible] when the rat renal medulla was [illegible] are [illegible]

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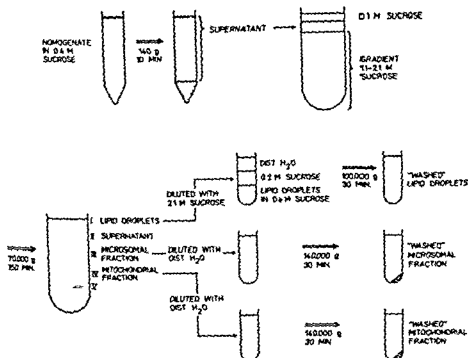


Fig 1 Procedure for fractionation of rabbit renal medulla by isopycnic gradient centrifugation The procedure for washing of the fractions is included in the diagram

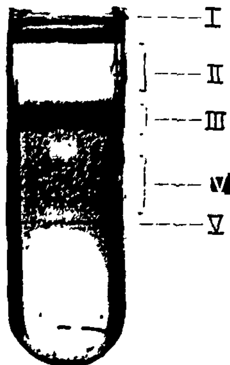
Fraction I, which had a volume of about 2 0 ml in each gradient was adjusted to a final sucrose concentration of 0 4 M by the addition of 2 1 M sucrose The adjusted solution was then transferred to centrifuge tubes fitting the SW 50 L rotor putting a maximum of 2 0 ml in each tube The fraction was gently overlayered with 1 5 ml 0 2 M sucrose and then 1 5 ml distilled water The tubes were centrifuged at 40 000 rpm (about 100 000 × g) for 30 min After the centrifugation a thin pellicle was present on the surface of the distilled water and was collected with a syringe Fraction III and IV which in each gradient had volumes of about 3 0 ml and 6 0 ml respectively were diluted to twice their volumes with distilled water and transferred to centrifuge tubes for rotor 50 They were centrifuged at 36 000 rpm (about 140 000 × g) for 30 min to form pellets at the bottom of the tubes The pellets were resuspended in 0 4 M sucrose All handling of the fractions was performed in a cold room at about +4° C

Protein was measured in the fractions by the method of Lowry et al (1951) with BSA as a standard after correction of the volume to [illegible] ml The protein content of the subcellular fractions were for lipid droplets < 0 08 mg supernatant [illegible]8 ± 3 2 mg microsomes 0 [illegible]8 ± 0 39 mg and mitochondria 0 27 ± 0 14 mg The whole homogenate contained 6 1 ± 1 2 mg ml

Electron microscopy In some experiments the subcellular fractions were fixed and prepared for electron microscopy immediately after collection [illegible] from the gradient In other experiments the frations were fixed after the washing procedure [illegible] described above

Fraction I was diluted to twice its volume with 1 % osmium tetroxide in 0 029 M veronal acetate buffer The other fractions were fixed by the addition of [illegible] % glutaraldehyde in 0 1 M cacodylate buffer pH 7 2 to obtain a final concentration of 1 5 % Fraction I was fixed for 60 min and the other fractions for 10 h All fractions were prepared for electron microscopy as previously described Bohman and Maunsbach 1971) and were examined in a Siemens Elmiskop I

Determination of prostaglandins in the papilla To see how much PGE_2 was present endogenously and how much was formed during the dissection and preparation of the subcellular fractions two different procedures were employed



used for biochemical analysis are labelled I IV Magnified 1 3 ×

a) The kidney was directly transferred from the anesthetized rabbit into liquid nitrogen The time required was 1 -2 s The frozen kidney was homogenized in chloroform -methanol (2 1) and lipid extract prepared according to Folch, Lees and Sloane Stanley (1957) The

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M potassium phosphate buffer pH 7 4 containing 3 mM reduced glutathione, 60 mM EDTA 0 1 % bovine serum albumin and 0 03 µg ³H labelled arachidonic acid Incubation was performed at 37° for 30 min In each experiment a control of whole homogenate and boiled homogenate was included Incubations were terminated by the addition of 9 volumes of 96 %

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urn with 30 ml of ethyl acetate benzene (1 9),
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taken up in 5 volumes of n heptane The n heptane was reduced to dryness under nitrogen and redissolved in 0 1—0 5 ml heptane prior to gas chromatographic analysis Recoveries of ^{3}H arachidonic acid to the nonpolar fraction were 90 4 ± 6 3 % (mean ± SD n = 9) in this procedure

The composition of the fatty acid esters in the sample was investigated using gas liquid ...

... of retention times with authentic compounds and by mass spectrometric analysis using a LKB 9000 combined gas chromatograph mass spectrometer equipped with a 1 % 6 foot SE 30 column The energy of the electrons was 22 5 eV

Quantitative measurements of the amount of arachidonic acid in the nonpolar and polar lipid fractions were performed with the aid of heptadecanoic acid as an internal standard This acid was combined with the sample prior to the injection A 1 % SE 30 column was used since this gave a better separation from the methyl ester of the saturated C_{22} acid (behenic acid) The temperature was programmed from 160° to 235° at a rate of 5°/min with a 5 min isothermal delay

Determination of 15-hydroxy prostaglandin dehydrogenase The renal cortex outer medulla and inner medulla (papil' ...
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The amount of 15 hydroxy prostaglandin dehydrogenase present in the pellet and in the supern ...

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Group separation of methyl esters of prostaglandins on Sephadex LH 20 was run on a 4×400 mm column as described previously (Anggård and Bergkvist 1970). For identification of prostaglandins by gas liquid chromatography with electron capture detection followed the principle described by Jouvenaz *et al* (1970) PGE_2 was converted to PGB_2 in 0.5 N NaOH. After acidification and extraction into ethyl acetate and evaporation the methyl ester was prepared using diazomethane and PGB_2 methyl ester was isolated by chromatography on Sephadex LH 20. The trimethylsilylether (TMSiO) was prepared in 25 µl each of pyridine and bismethyl trimethylsilyl acetamide (BSA, Pierce Chemical Company, Rockford Ill USA). After 30 min at room temperature the sample was evaporated to dryness in an evacuated desiccator and the material was redissolved in 0.5 ml of dry toluene. Gas chromatographic conditions 1% OV 1 on 100—200 mesh Gaschrom Q run at 235° in a Hewlett Packard model 402 gas liquid chromatograph equipped with a ^{63}Ni electron capture detector. The detector temp was 250° C. The pulse interval to the detector was set to 50 µs.

Results

Electron microscopy of the isolated subcellular fractions The ultrastructural appearance of the subcellular fractions from rabbit renal papilla was in good agreement with the earlier observations on fractions from rat renal medulla (Bohman and Maunsbach 1971). The pellet obtained at 140 *g* consisted to a large extent of basement membrane like material and nuclei. Large cell fragments containing various cell organelles were also found.

Fraction I, the lightest fraction contained round granules or droplets with a homogenous structure less matrix and a dense marginal zone. The size of the granules varied but the majority of the granule profiles were 0.2—0.5 µ in diameter. The granules were morphologically indistinguishable from the lipid droplets in the interstitial cells of the renal medulla. Very little contaminating material was found in this fraction. In the fraction that had been washed the granules were somewhat irregular (Fig 3). Sometimes large granules appeared to the result of fusion of several small granules.

The supernatant (fraction II) contained only a very small amount of sedimentable material. Some membrane fragments and occasional mitochondria were found but the bulk of this material was amorphous and could not be morphologically identified.

Fraction III (Fig 4) was morphologically a microsomal fraction consisting predominantly of small vesicles and membrane fragments with or without ribosomes attached. Most of the vesicles and fragments consisted of membranes of a thin type, 60—70 Å, as the membranes of the endoplasmic reticulum of intact cells. Some membranes however, were of a thick type 90—100 Å similar to plasma membranes of cells. The majority of the vesicles had a diameter which ranged between 0.05—

Fig 3 Elect[illegible] fraction I after osmium tetroxide fixation [illegible] s a most exclusively of round lipid granules [illegible] The granules have a dense marginal zone [illegible]

Fig 4 Electron micrograph of microsoma[illegible] III after washing glutaraldehyde fixation and sedimentation. The fraction con[illegible] mainly of membrane fragments and vesicles. Most membranes have a thickness of about 60—[illegible]0 Å but some are of another type with thickness of about 90—100 Å. Many ribosome[illegible] (R) are also present either free or attached to membranes. Some dense, lysosome-like b[illegible] (L) are also seen. × 45 000



Fig. 5 a. Electron micrograph of mitochondrial fraction (IV) after washing glutaraldehyde
fugation and sedimentation. The fraction consists predominantly of mitochondria (M) most
of which are swollen and empty. Many lysosome like bodies (L) are seen. Membrane fragments
of different thickness are also present. Many of the thin membranes have ribosomes attached.
× 20 000

Fig. 5 b. Higher magnification of the fraction seen in Fig. 5 a showing mitochondria in vary-
ing degrees of preservation. No. 1 seems to be morphologically well preserved whereas no. 2
and 3 are swollen with an empty matrix. The 2 surrounding membranes are however visible
arrows. × 50 000

0.3 μ. Many free ribosomes were seen. Occasional dense membrane bound bodies similar in structure to lysosomes were also observed. The washing procedure caused no apparent changes in the ultrastructure of the fraction (Fig. 4).

Fraction IV contained mainly mitochondria some of which appeared swollen with an empty matrix. Some membrane fragments and vesicles were also found as were dense membrane limited bodies presumably lysosomes similar to those in the microsomal fraction. The normal ultrastructure of the mitochondria was not well preserved in the fraction which had been washed and although still possible to identify as mitochondria the majority were swollen, empty or sometimes disrupted (Fig. 5 a, b).

The thin red band (V) located below fraction IV in the gradient consisted of red blood cells and mitochondria.

TABLE I Inhibition of prostaglandin biosynthesis in homogenates of rabbit renal papilla

Exp	PGE₂ concentration µg/g		
	Synthesis inhibition	No synthesis inhibition	Increase in PG during preparation of subcellular fractions
1	4 8	14 0	2 9
2	23 5	44 5	1 9
3	24 0	39 5	1 6
Mean	17 0	32 7	2 0

The pellet at the bottom of the gradient consisted principally of the same components as the pellet obtained at 140×g

Occurrence of prostaglandins in the subcellular fractions In three experiments the smooth muscle stimulating activity in fractions I—IV was studied The results are shown in Fig 6 Most of the activity (68—70 %) was recovered in the supernatant fractions Smaller amounts were found in the microsomes (14—23 %) and mitochondria (7—19 %) As described below it could be shown that essentially all of the biological activity was due to prostaglandin E_2 (PGE_2)

In separate experiments was studied how much PGE_2 was present endogenously and how much was formed during the dissection and centrifugation procedures respectively When the kidney was quick frozen by immersion in liquid nitrogen the content of PGE_2 was low 0 005 µg/g of tissue During the dissection and the time before centrifugation the level of PGE_2 rose over a 3000 fold to about 17 µg/g (Table 1) During the preparation of the subcellular fractions there was a further 2

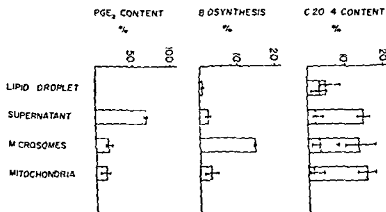


Fig 6 Distribution of PGE_2 prostaglandin biosynthesis (% of C20 4 converted to PG) and arachidonic acid (% of total fatty acids) (in nonpolar lipids ——— in polar lipids) in the subcellular fractions of the rabbit renal papilla. Each value is the mean and range of at least 3 expts

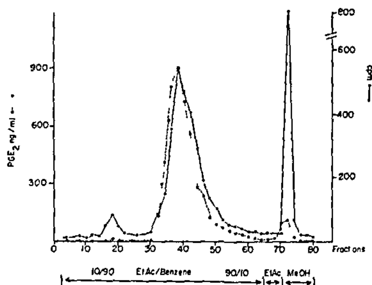


Fig 7 Silicic acid chromatography of the smooth muscle stimulating lipids from the rabbit renal papilla ^{3}H PGF_2 is included as a reference

fold increase in the PG level of PGE. The results thus show that the endogenous level of PGE_2 in the papilla is low but that high amounts are formed rapidly after e kidney is removed The formed prostaglandins are recovered mainly in the luble fraction of the homogenate (Fig 6)

Identification of the smooth muscle stimulating activity as PGE_2 When the lipid extract from the subcellular fractions was subjected to chromatography on silicic acid using gradient elution 95 % of the biological activity cochromatographed with added ^{3}H PGE (Fig 7) This material was subjected to fluorimetric enzymatic analysis using 15 hydroxy PGDH and PGE_2 as a reference 89 5 % of the smooth muscle stimulating activity applied on the column was accounted for in terms of PGE_2 equivalents Furthermore the active compound lost activity upon incubation with 15 hydroxy prostaglandin dehydrogenase (Table II)

TABLE II Inactivation of smooth muscle stimulating activity from rabbit renal papilla by 15 hydroxy prostaglandin dehydrogenase (PGDH)

Substrate	NAD^+ 2 mM	PGDH 0 2 mU/mg	Smooth muscle stimulating activity after incubation
0 1 ml of papilla extract	+	+	0
"	+	−	40 ng/ml
"	−	+	40 ng/ml
"	−	−	40 ng/ml
—	+	+	0

TABLE III Content of arachidonic acid (C20 4) in subcellular fractions of the rabbit renal papilla Each value is the mean ± S D Figures in brackets indicate number of experiments

Fraction	C20 4 μg/g papilla		C20 4 % of total fatty acids	
	nonpolar lipids	polar lipids	nonpolar lipids	polar lipids
Lipid droplets	27 9±10 0(3)	0 4±0 6(3)	5 1±2 2(6)	3 4±2 3(5)
Supernatant	8 9±2 3(3)	46 3±31 0(3)	2 3±1 4(6)	14 7±1 2(6)
Microsomes	4 5±2 9(3)	30 0±23 0(3)	3 2±3 1(6)	13 4±3 0(6)
Mitochondria	0 6±0.5(3)	49 1±10 0(3)	1 8±1 4(6)	15 4±1 6(6)

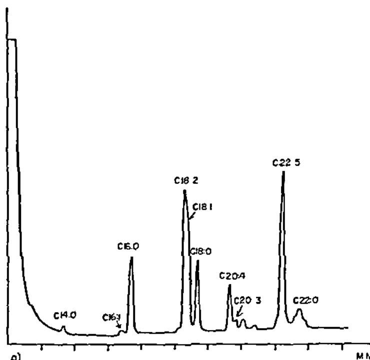
After conversion to the PGB-compound with methanolic KOH treatment with diazomethane and purification on a Sephadex LH 20 column the compound was analyzed by gas liquid chromatography and electron capture detection (Jouvenaz *et al* 1970) A single peak was observed with a rentetion time identical to that of the corresponding derivative of authentic PGE_2

The amount of PGE_2 present in the original sample could be calculated using isotope dilution of the tracer ^3H PGE_2 and plotting the peak height of the sample against standards of PGE_2-derivatives The value found was 75 % of that obtained by smooth muscle assay of the original lipid extract The result thus show that under the present conditions most of the smooth muscle stimulating activity could be accounted for by the presence of PGE

Biosynthesis of ^3H PGE_2 from ^3H arachidonic acid in subcellular fractions of the rabbit renal papilla The biosynthesis of PGE_2 was studied with the aid of ^3H arachidonic acid The prostagladin synthetase was to be found mostly in the microsomal fraction (Fig 6) Small conversions were observed also in the supernatant and mitochondrial fractions whereas no significant biosynthesis was found in the lipid droplets

The metabolism of prostaglandin E_2 in the rabbit kidney The amount of 15 hydroxy prostaglandin dehydrogenase (PGDH) was measured in the particulate and nonparticulate fractions of the rabbit kidney No demonstrable PGDH activity was found in the papilla Under identical conditions significant conversions to 15 keto metabolites were observed in the nonparticulate fraction from the outer medulla and cortex

Composition of fatty acids in lipids of the rabbit renal papilla The relative abundance of the major fatty acids in the polar (phospholipids, sphingolipids cereamides) and nonpolar (glycerides cholesterol esters and free fatty acids lipids of the subcellular fractions were studied by means of gas liquid chromatography The result is shown in Figs 6 and 8 It is seen that arachidonic acid is by far the major unsaturated C_{20} acid in the polar lipids, amounting to 15 % of the total fatty acid content in microsomes supernatant and mitochondria Less arachidonic acid was found to be present in the polar lipids from the lipid droplet fraction about 3 % In a



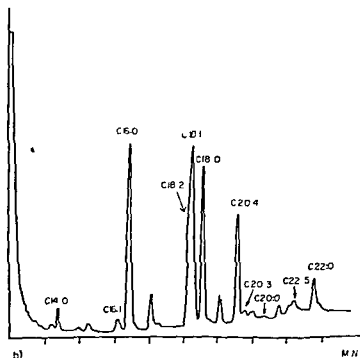


Fig 8 Gas liq[illegible] fatty acid composition of a) nonpolar li[illegible] polar lipids in microsomal fraction from [illegible] perature program 5 min delay at 160°

subcellular fractions less arachidonate was found in the nonpolar as compared to the polar lipids, except in the lipid droplet fractions (Table III) The absolute amounts of arachidonic acid in the subcellular fractions are shown in Table III It is seen that the arachidonic acid occurs most abundantly in the non polar lipids of the lipid droplet fraction and in the polar lipids of the supernatant, microsomes and mitochondria

The lipid droplets consisted of about five times more non polar than polar lipids as compared to a ratio of about 1 1 for the other subcellular fractions The fatty acid composition of the nonpolar lipids of the lipid droplets was furthermore unique in having high (10 %) relative abundance of docosapentaenoic acid (Fig 8, a) The identity of this acid was established by its gas chromatographic and mass spectrometric properties The retention times on the 8 % EGSS X column and on a column of 1 % SE-30 agreed with the expected position of the C_{22} acid having five double bonds The methyl ester was isolated by preparative gas chromatography and subjected to catalytic reduction in deuterium or hydrogen using Adams catalyst (PtO_2) The products had retention times identical to that of behenic acid (C_{22}) methyl ester The molecular ion of the ester reduced in hydrogen had a m/e value of 354, M for $C_{..}$ and the ester reduced in deuterium a m/e value of 364 The results show that this fatty acid is docosapentaenoic acid

Discussion

Previously prostaglandin synthetase have been localized to the microsomal fraction of sheep vesicular glands (Hamberg and Samuelsson 1967), rat adipose tissue (Christ and Nugteren 1970) The subcellular distribution of prostaglandins have been studied in rat (Kataoka, Ramwell and Jessup 1967) and rabbit brain (Hopkin, Horton and Whittaker 1968) with divergent results The 15 hydroxy prostaglandin dehydrogenase and prostaglandin Δ^{13} reductase have been localized mainly to the high speed supernatant fractions of swine lung spleen and kidney (Änggård and Samuelsson 1964, Änggård Larsson and Samuelsson 1971) The present work represents an attempt to a comprehensive study of the subcellular localization of substrates and enzymes of the prostaglandin system in one tissue namely the rabbit renal papilla

Our results show that also in the rabbit renal papilla the prostaglandin synthetase is localized mainly to the microsomal fraction As judged by electron microscopical findings this fraction contained mainly smooth and rough surfaced vesicles limited by thin membranes which were apparently derived from the endoplasmic reticulum and membrane fragments of thick membranes presumably derived from cell membranes It is not possible at the present time to decide which of these cell components that contain prostaglandin synthetase, nor is it known if the enzyme system is present in one or several types of cells of the inner medulla The rate of biosynthesis was relatively high confirming previous work by Hamberg (1969)

In agreement with van Dorp (1971) the level of prostaglandin E_2 was found to be high about 17 μg/g of tissue a value paralleled only by the concentrations in human seminal fluid It should be pointed out, however, that nearly all was formed during the dissection of the papilla and that the *in vivo* concentrations are much lower

The formed prostaglandins were recovered mostly in the supernatant fractions This is in agreement with that previous work on rabbit brain homogenates where most of the prostaglandin like material was found in the nonparticulate part of the homogenate (Hopkin Horton and Whittaker 1968) No evidence for retention or concentration of prostaglandin within subcellular particles were found It thus seems that after or in connection with their formation the prostaglandins are released from the endoplasmic reticulum or the plasma membranes into the cytoplasm or to the extracellular space

The level of arachidonic acid in the papilla was about 200 μg/g Most of the arachidonate was found in the polar lipids of the microsomal mitochondrial and supernatant fractions In the lipid droplets very little arachidonate was found in the polar lipids whereas appreciable amounts were seen in the nonpolar lipid fraction In the rat these lipids consist of triglycerides cholesterol esters and free fatty acids in the approximate ratio of 3 2 1 (Nissen and Bojesen 1969) Since the amount of prostaglandin E_2 formed in the papilla was about 50 μg/g it appears that a considerable part of the totally available arachidonate in the papilla can be utilized for prostaglandin formation Similarly the amount of prostaglandins formed nearly ed the amounts of arachidonate found in the microsomes the site of synthesis Therefore a considerable portion of the phospholipid bound arachidonate in this fraction should be available for prostaglandin synthesis It can however not be excluded that some prostaglandin precursors are mobilized from other subcellular compartments

It has been suggested (Nissen and Bojesen 1969) that the arachidonate esterified to the triglycerides in the lipid droplets would serve as a prostaglandin E_2 precursor store However this hypothesis does not fit with the current well documented concept (Samuelsson 1969 Kunze and Vogt 1970) that an activation of a phospholipase A is the rate limiting initial step in the prostaglandin biosynthesis The preferential utilization of locally available esterified precursor rather than the labelled exogenous free acid is moreov r upported by recent studies in homogenates of rat stomach (Pace Asciak and Wolfe 1 70)

Low levels of 15 hydroxy prostaglandin dehydrogenase was found in the inner medulla Histochemical studies on the distribution of the 15 hydroxyprostagland n dehydrogenase in the rat kidney (Nissen and Andersen 1968) indicate that the activity is mostly associated with the ascending limb of the loop of Henle and in the distal tubule whereas only small activity is found in the renal capillaries and veins and in the inner medulla Thus prostaglandin formation is maximal in the medulla as compared to the cortex (Crowshaw and Szlyk 1969) whereas the converse is true for inactivation by the 15 hydroxy prostaglandin dehydrogenase (Anggård Larsson

and Samuelsson 1971) Thus it seems that under physiological conditions prostaglandins formed in the renal papilla may either have a local effect in the papilla and/or may leave the inner medulla by way of the vasa recta without undergoing metabolic inactivation possibly to act on juxtamedullary structures

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Acta physiol. scand 1972. 84 247—253

From the Max Planck Institut für Experimentelle Medizin, Arbeitsgruppe Neurochemie, Gottingen Germany, and from the Department of Histology, University of Umeå, Umeå, Sweden

Microdetermination of Free Amino Acids in Pancreatic Islets Isolated from Obese-Hyperglycemic Mice

By

GUNTER BRIEL, ERIK GYLFE, BO HELLMAN and VOLKER NEUHOFF

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Abstract

BRIEL, G, E GYLFE, B HELLMAN and V NEUHOFF *Microdetermination of free amino acids in pancreatic islets isolated from obese hyperglycemic mice* Acta physiol scand 1972 *84* 247—253

Several amino acids are potent stimulators of insulin release from the pancreatic β-cells (Fajans *et al* 1967, Lambert *et al* 1969 Milner 1970) This together with the fact that insulin is a protein, makes amino acid metabolism an interesting aspect of β cell physiology

So far there seem to have been no studies to characterize the pool of free amino acids in the pancreatic β-cells This is certainly due to the methodological difficulties involved in experimentation with the minimal amounts of tissue represented by pure mammalian islets In the work reported here this problem has been approached with a microprocedure involving ^{14}C labelled dansyl Cl (1 dimethylaminonaphthalene sulfonyl chloride) and two dimensional thin layer chromatography This allows quantitation of amino acids in the range of 10^{-12} mol (Neuhoff *et al* 1969) The application of the technique to microdissected islets of obese hyperglycemic mice made it possible to obtain data representative for the insulin secreting β-cells (Hellman 1965, 1970)

Materials and Methods

Isolation of tissues and incubation conditions Male 7 month old obese hyperglycemic mice (gene symbol *obob*) from the Umeå colony (Hellman 1965) were starved overnight The animals were killed by decapitation and serum samples taken Fresh pancreatic islets and equally sized pieces of exocrine parenchyma were isolated by free hand microdissection (Hellerstrom 1964) in Krebs Ringer bicarbonate buffer supplemented with 0 6 mg/ml glucose and equilibrated with 95 % O_2+5 % CO_2 The samples of endocrine and exocrine pancreas were incubated for 60 minutes at 37° C in fresh dissection medium After freeze drying overnight at −40° C and 0 001 mm Hg the specimens were weighed on a quartz fibre balance (Lowry 1953) For each animal the analyses included 3—5 islets or a similar number of specimens from the exocrine pancreas

Preparation of tissue extracts Deionized distilled water (2° C) was added to the freeze dried tissue specimens in a small test tube to a final concentration of 4—8 μg tissue per μl Three successive freezings and thawings were followed by sonication (Ultrapoint 40 WST *Oscillator, 32 kHz Ultrapoint Ltd Uppsala Sweden) for five minutes and another three*

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centrifugation 4 μl samples of the islet or exocrine pancreas supernatants were transferred to a cone shaped reaction vial with a total volume of 300 μl (Reacti Vial, Pierce Chemical Company Rockford Illinois U S A) and dried under vacuum A 10 μl sample of the serum supernatant was also dried 2 μl 0 05 M $NaHCO_3$ was added to the dried samples of endocrine and exocrine pancreas The corresponding volume added to the serum samples was 50 μl which yielded extracts diluted 10 times Two μl samples from the serum extracts were transferred to Reacti Vials For all types of extracts the $NaHCO_3$ solution was adjusted to give pH 10 0 after mixing

Preparation of amino acid standards A mixture was prepared in 0 05 M $NaHCO_3$ of all amino acids analysed (pH adjusted to 10 0) in approximately the same proportions as present in the pancreatic islets The total concentration of groups reactive with dansyl-chloride never exceeded 1 9 mM The amino acid concentrations varied from 0 0? mM (tryptophan) to 0 76 mM (taurine) Serial dilutions were performed to give 20 40 60 80 and 100 % of the initial concentration A 2 μl sample of each concentration was transferred to Reacti Vials

Dansylation conditions The procedure of dansylation was based on the work of several authors as reviewed by Seiler (1970) also paying attention to a recent work by Spivak *et al* (1971) Four μl 10 mM ^{14}C labelled dansyl Cl in acetone (C E A 91 Gif sur Yvette France) was added to each Reacti Vial The vials were immediately closed and incubated for 30 min in a dark 37° C water bath After incubation the reaction mixtures were dried under vacuum

Chromatography Each dried sample was dissolved in 2 μl acetic acid acetone (2 3 Woods and Wang 1967) A small calibrated capillary was used to apply 250 nl of the mixture to a 5×5 cm polyamide thin layer plate (TLC Ready Plastic Sheets F 1700 Micropolyamide Schleicher & Schull Dassel Germany) Two-dimensional chromatography was then performed using modifications of the Hartley (1970) procedure as described by Neuhoff *et al* (1971) The solvents used and other details are given in the legend to Fig 1 After separation the fluorescent spots of the dansylated amino acids were marked with a soft pencil under ultra violet light and scraped off for radioactive measurements (Neuhoff *et al* 1970 a and b)

Measurements of radioactivity The dansyl amino acids scraped from the chromatograms were transferred to vials containing 10 ml scintillation liquid (4 g PPO and 100 mg POPOP per liter toluene) Counting was performed in a Packard scintillation spectrometer (Model 3375) The tissue content of an individual amino acid was calculated from its linear standard curve as exemplified in Fig 2 It was assured that the tissue concentrations used were within the capacity of the technique for all amino acids analysed (Fig 3)

Results

14 amino acids were subjected to quantitative analysis (Table 1) Twelve of these were present in amounts large enough to be measured in the pancreatic islets Arginine cystine, glutamine methionine serine threonine and tyrosine were also identified in the islet specimens but could not be measured due to incomplete chromatographic separation In addition to the above mentioned amino acids the islets contained trace amounts of histidine

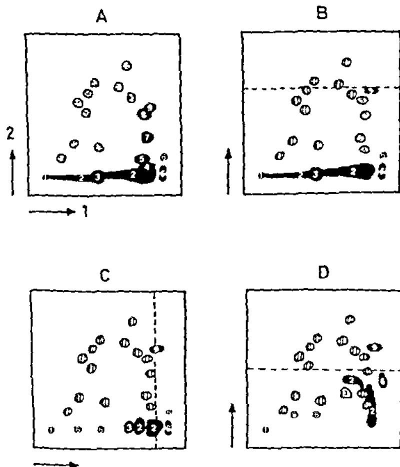


Fig 1 Ch[illegible] 5 × 5 cm) The [illegible] ting constantly [illegible] the solvents w[illegible] acid (9 1) *B* ethyl acetate methanol acetic acid (20 1 1) *C* and *D* 3 % formic acid Spots indicated (●) are not sufficiently separated those individacted (○) are ready to scrape off Spots indicated (◎) refer to holes remaining after the fluorescent spots have been scraped off As can be seen 10 spots were removed after the first 2 separations – *A* After separation *B* run to the broken line another 4 spots were scraped off Separations *C* and *D* were performed to separate dansyl taurine from the by product dansyl OH The fronts were allowed to run as far as indicated by the broken lines The spots are indicated as follows 1 starting point 2 = dansyl-OH 3 = dansyl taurine, 4 = dansyl-aspartic acid 5 – dansyl glutamic acid 6 = unknown by-prod[illegible] 9 dansyl NH_2 10 dansyl gamm[illegible] oline 3 = dans[illegible] isoleucine 14 = [illegible] enylalanine 1 bis-dansyl lysine

It is evident from Table I that the islets have a much larger amino acid pool than the exocrine pancreas For the 14 amino acids listed in the Table the total values were 102 and 47 mmol per kg dryg weight respectively In both endocrine and exocrine pancreas about 50 [illegible]

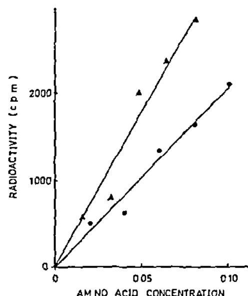


Fig 2

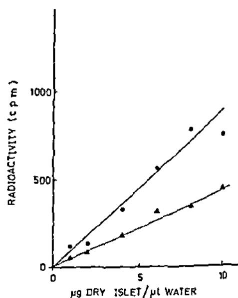


Fig 3

Fig 2 Standard curves for dansyl alanine (●) and bis dansyl lysine (▲) Note the higher radioactivity of the bis compound

Fig 3 Radioactivity measured after dansylation of serial dilutions of a concentrated islet extract The linear relationship is exemplified with the same amino acids as in Fig 2

TABLE I Tissue contents of free amino acids in obese hyperglycemic mice The levels are given in mmol/kg dry weight for the pancreatic islets and exocrine pancreas and in mM for serum 6 animals were used for the analyses of the islets and 3 animals for the exocrine pancreas and the serum The figures refer to mean values±S E M GABA gamma aminobutyric acid.

Amino acid	Pancreatic islets	Exocrine pancreas	Serum
Alanine	*5 4±0 5*	*1 5±0 3*	*0 123±0 016*
Aspartic acid	10 2±0 4	1 1±0 3	—
GABA	2 1 0 4	—	—
Glutamic acid	12 6−0 5	5 3±0 3	0 032±0 016
Glycine	7 3±0 4	9 0±1 0	0 072±0 003
Isoleucine	Trace	0 1+0 1	0 047±0 006
Leucine	4 8±0 9	0 4±0 3	0 088±0 009
Lysine	2 2±0 2	0 9 0 2	0 046±0 005
Ornithine	0 3+0 2	0 5±0 3	0 030±0 005
Phenylalanine	0 4±0 2	0 4+0 2	0 048±0 004
Proline	1 5±0 1	0 7±0 1	0 054±0 004
Taurine	51 6−3 8	26 4±3 5	0 530±0 067
Tryptophan	Trace	—	0 069±0 014
Valine	3 8±0 4	0 3±0 2	0 100±0 010

case for the free amino acid pattern of the serum As compared to the exocrine pancreas, the islets contained a tenfold excess of aspartic acid, leucine and valine The presence of gamma aminobutyric acid (GABA) was another characteristic feature of the islet amino acid pool

Discussion

Measurements of free amino acids by means of dansylation are complicated by the fragmentation reaction as described by Neadle and Pollitt (1965) Another technical problem is concerned with the fact that certain amino acids such as histidine, ornithine and lysine form bis dansyl compounds In the present study several dilutions of a reference mixture containing the amino acids analysed were dansylated at the same time as the tissue samples This made it possible to obtain at least semi quantitative estimates of the amino acid content of the tissues (Crowshaw *et al* 1967, Seiler 1970) Since the concentrations of the amino acids in the reference mixture were adjusted to the islet levels recordings for the islet should be regarded as more reliable than those obtained for exocrine pancreas and serum

To obtain results representative for the insulin secreting β cells the islets were microdissected from the pancreas of obese hyperglycemic mice These islets contain more than 90 % β-cells (Hellman 1965), which respond adequately to all secretagogues tested including amino acids such as leucine and arginine (Lernmark 1971 a b) With the same experimental model it has been demonstrated that the β cells are equipped with mechanisms for concentrative uptake of neutral and dibasic amino acids similar to those found in other types of mammalian cells (Hellman *et al* 1971 a b Christensen *et al* 1971) The present data reflect the situation in β cells ready for a prompt response to various insulin secretagogues other amino acid levels might be expected for islets in equilibrium with exogenous amino acids as is the case with serum during *in vivo* conditions

Tallan *et al* (1954) reported in detail the distribution of free amino acids among various tissues in the cat These authors noted relatively high concentrations of most amino acids in the pancreas The present investigation showed that the islets contained more free amino acids per unit weight than the exocrine pancreas While the insulin molecule lacks tryptophan this amino acid constitutes as much as 5 3 % of the glucagon molecule (Bromer *et al* 1956 The finding of only trace amounts of tryptophan *in the islets might therefore be attributable to the fact that they contain* very few of the glucagon producing α cells The observation of higher glutamate levels in the endocrine than in the exocrine pancreas is consistent with recent studies on obese hyperglycemic mice employing fluorometric microtechniques with glutamate dehydrogenase (Danielsson *et al* 1970) The use of a different extraction procedure employing heating with dilute HCl may well explain the somewhat higher values obtained with the enzymatic approach

It is tempting to assume that the very high level of islet taurine is related to the specific role of the β-cells in synthetising a sulfur containing hormone The fact that

taurine is an oxidation product of cysteine poses the question whether it may represent a degradation product of insulin For example, it is possible that insulin is synthetized in excess and subjected to intracellular degradation if stimuli for its release are inadequate The high cell levels of taurine could, however, also reflect a more general phenomenon in our experimental animals since this amino acids was also abundant in the exocrine pancreas and the serum. The serum levels of taurine measured are 10 times higher than those found in the rat (Roberts 1968)

The brain is considered to contain particularly large amounts of taurine (White, Handler and Smith 1970) Another similarity between the β-cells and the nervous system appeared from the demonstration of substantial amounts of GABA in the islet specimens Subcellular fractionation studies of the mouse brain have shown this amino acid to be particularly associated with nerve ending fragments (Weinstein *et al* 1963) The sparse innervation of the pancreatic islets in rodents (Falk and Hellman 1963 Lacy 1960), however, makes it rather unlikely that the nervous component account for the high islet levels of GABA

Leucine and arginine, when present in concentrations of 5 and 10 mM respectively, markedly stimulate insulin release from islets microdissected from obese hyperglycemic mice (Lernmark 1971 a and b) The ability of these amino acids to stimulate insulin release made it of interest to determine their contribution to the amino acid pool of the β-cell While technical factors made it impossible to obtain quantitative information about arginine, we found no less than 4 8 mmol leucine per kg islet dry weight or 10 times as much as in the exocrine pancreas This is a surprisingly large amount, especially since the rapid equilibration of leucine across the β-cell membrane (Hellman *et al* 1971 b) could be expected to dramatically lower its intracellular content during incubation in the absence of amino acids In the light of the present data it therefore seems plausible to assume that the β-cells are equipped with sites for binding large amounts of leucine It should be a matter for further study to determine whether such leucine binding generates a signal for insulin release

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Acta physiol. scand. 1972. 84. 254—260

From the Department of Pharmacology, Royal Veterinary College Stockholm and the Department of Toxicology, University of Uppsala, Sweden

Uptake of ³H-Atropine in the Pancreatic Islets and the Parafollicular Cells of the Thyroid in Mice Shown by Microautoradiography

By

PREMYSL SLANINA and HANS TJÄLVE

Received 12 August 1971

Abstract

SLANINA, P and H. TJÄLVE. *Uptake of ³H-atropine in the pancreatic islets and the parafollicular cells of the thyroid in mice shown by microautoradiography* Acta physiol scand. 1972 *84* 254—260

Male albino mice were injected with ³H atropine and killed 5 min, 30 min and 2 h after the injection The distribution of radioactivity in the pancreas and the thyroid was then studied with a microautoradiographic technique for isotopes in water soluble form In the pancreas the highest radioactivity was found in the pancreatic islets In the thyroid the radioactivity was most strongly accumulated in cells with a distribution corresponding to the parafollicular cells A method for a combination of monoamine fluorescence with microautoradiography was also used to show that the cells of the pancreatic islets and the parafollicular cells of the thyroid which are capable of monoamine accumulation after monoamine precursor injection also are capable to accumulate atropine

Albanus *et al* (1968) have recently, using whole-body autoradiography demonstrated that injected atropine is accumulated in some endocrine organs such as the pancreatic islets and the thyroid In order to obtain a more detailed information on the cellular localization of atropine in these endocrine organs, we have performed a microautoradiographic study in mice using ³H atropine

As it also has been shown that these cell systems are able to store monoamines after injection of monoamine-precursors (see Ritzen *et al* 1965, Larsson *et al* 1966 Gershon and Ross 1966, Cegrell 1968), a comparison was made between the localization of atropine and dopamine, formed after the injection of DOPA For this purpose a combination of microautoradiography and monoamine fluorescence was employed.

Material and methods

Animals

Male albino mice of NMRI strain (body weight 20 g) have been used Th animals were kept on a complete pellet diet (AB Ewos, Sweden) at a room temperature +25° C and were given water ad lib

Compounds

^{3}H atropine (spec act 0 5 Ci/mmol, The Radiochemical Centre, Amersham England) was dissolved in diluted HCl (5 mCi in 1 0 ml 0 05 N HCl) Non labelled L-ß-3, 4-didhydroxy phenylalanine (DOPA) was obtained from SIGMA Chemical Company, USA

Microautoradiography

6 mice were injected into a tail vein with 0 5 mCi ^{3}H atropine each (corresponding to 20 mg/kg b w expressed as atropine sulphate) 5 min, 30 min and 2 h, respectively, after the injection the mice were killed by decapitation (two mice at each time interval) and small pieces of tissue from the pancreas and thyroid were quickly removed and immediately frozen in isopentane cooled with liquid nitrogen

The pieces were then freeze-dried under vacuum (10^{-4} mm Hg) at about −40° C for 5 d [illegible]

Combined microautoradiography and fluorescence microscopy

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−20° C for about 2 months Thereafter the tape was removed in xylol and the photographic plates were developed (Hammarstrom *et al* 1965) After staining with hematoxylin and eosin and mounting in Canada balsam it was possible to compare the autoradiographic pictures with the previously obtained fluorescence pictures of the same areas

To ascertain that no radioactivity had been redistributed during the embedding procedure pieces of paraffin near the embedded tissue were dissolved in Soluol (Packard) and counted in a scintillator No activity could be detected in the paraff n

Results

Thyroid

In the thyroid a large number of strongly labelled cells appeared after the injection of ^{3}H atropine (Fig 1) The labelled cells were situated in clusters between the follicles or basally within the follicular lining with a distribution pattern indicating that they were identical with the parafollicular cells The silver grains tended to be distributed diffusely over the whole cell Only a slight radioactivity could be observed in the

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At each time interval several sections were also subjected to conventional stripping film autoradiography which included deparaffinization in xylol and alcohol and floating of AR−10 (Kodak) stripping film on the sections in water

Combined microautoradiography and fluorescence microscopy

Since atropine is present in the tissues in a water soluble form a method was developed for the combination of the histochemical method of Falck and Hillarp for the visualization of biogenic amines (see Falck and Owman 1965) with a microautoradiographic method for isotopes in water soluble form (Hammarstrom *et al* 1965)

Thus two mice were injected intravenously with non labelled DOPA (100 mg/kg in 0 2 ml distilled water) 1 1/2 h later they received 0 5 mCi ^{3}H atropine i v each 30 min after the ...

... and put on warm (+60° C) slides for about 30 sec allowing the paraffin to melt and the sections to spread

After examination and photographing of dopamine fluorescence in the pancreatic islets and the thyroid the paraffin of the sections was again allowed to melt by brief warming A piece of cellophane tape was then placed over the section and carefully pressed against the slide close to the section. When the tape was lifted the section came off adhering to the tape The section carrying tape was then fastened to photographic plates (Ilford K2 or G5) and exposed at −20° C for about 2 months Thereafter the tape was removed in xylol and the photographic plates were developed (Hammarstrom *et al* 1965) After staining with hematoxylin and eosin and mounting in Canada balsam it was possible to compare the autoradiographic pictures with the previously obtained fluorescence pictures of the same areas

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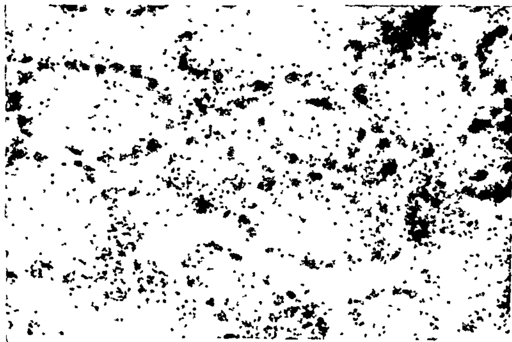


Fig 1 Microautoradiogram of the thyroid of a mouse 30 min after the injection of ^{3}H atropine A strong accumulation of radioactivity can be seen in some cells with a distribution pattern indicating that they are identical with parafollicular cells Ilford G5 nuclear plate Htx eosin ×560

colloids and the follicular cells The labelling appeared already 5 min after the injection of ^{3}H-atropine and persisted for 2 h After 2 h, however, the uptake of activity was very weak compared with the shorter time intervals

After the combination of microautoradiography with the histochemical fluorescence technique, enabling the visualization of catecholamine-containing parafollicular cells (Falck *et al* 1964, Larsson *et al* 1966), it was demonstrated that the intensely labelled cells corresponded to the fluorescing parafollicular cells (Fig 2)

In the sections, which were deparaffinized and passed through alcohol to distilled water, before the application of photographic film there was a random activity spread out all over the tissue, without any sign of localization to specific cells

Pancreas

In the pancreas a marked activity could be seen in the islets of Langerhans 5—30 min after the injection of ^{3}H-atropine (Fig 3) The activity was rather uniformly distributed throughout the islet with most of the cells being labelled Silver grains could be seen both over the nucleus and the cytoplasm of the cells After 30 minutes the labelling subsequently disappeared Some labelling was observed in the exocrine

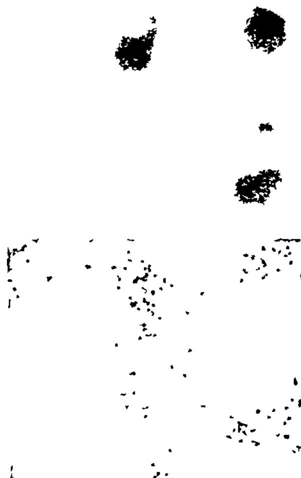


Fig 2 a Fluorescence photomicrograph of a section from the thyroid of a mouse 2 h after the injection of non labelled DOPA 3 fluorescing parafollicular cells are seen

Fig 2 b Microautoradiogram of the same section obtained through the injection of ^{3}H atropine 1 1/2 h after the injection of non labelled DOPA The fluorescence in the parafollicular cells is in accordance with the accumulation of silver grains Ilford K2 nuclear plate Htx eosin × 480

pancreas, but it was always considerably lower than in the islets The combination of fluorescing microscopy and microautoradiography after injection of DOPA and tritium labelled atropine to the same mice showed that the radioactivity was present in all the fluorescing cells of the islets

A diffuse distribution of the silver grains throughout the whole pancreas without any specific localization could be seen in the section which had been deparaffinized and subjected to conventional stripping film technique

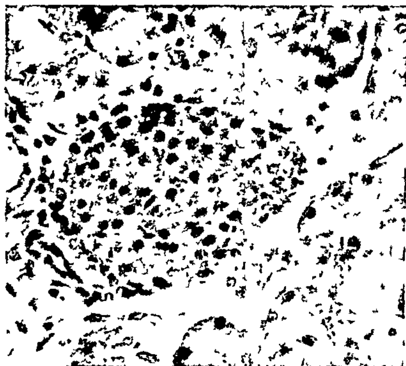


Fig 3 Microautoradiogram of a pancreatic islet of a mouse 30 min after the injection of ^{3}H atropine The activity is distributed throughout the whole islet Ilford K2 nuclear plate H x eosin ×480

Discussion

The autoradiograms described in this paper show that after the injection of ^{3}H atropine into the mice the radioactivity within the pancreas is accumulated mainly in the islets In the thyroid the highest concentrations of silver grains are confined to the parafollicular cells which are known to produce thyrocalcitonin in mammals (Bussolati and Pearse 1967 Kracht *et al* 1968 Kalina *et al* 1970) This is in accordance with the findings by Albanus *et al* (1968) who used a whole body autoradiographic technique No attempt has been made in the present paper to establish the identity of radioactivity in the studied organs but Werner (1969) while studying the distribution and metabolism of ^{3}H atropine in the mouse found about 50 % of atropine in the pancreas unchanged within 30 min after the injection Additionally there were relatively very low levels of atropine metabolites in the various organs of the mouse except in the liver (Werner 1969 Albanus *et al* 1968)

Both the pancreatic islet cells and the parafollicular cells belong to the group of cells with several common cytochemical and ultrastructural characteristics which are known to or sugegsted to secrete a low molecular weight polypeptide hormone (for review see Pearse 1969) They were distinguished by the term APUD cells (Pearse 1968), as one of their common characteristics is ability to synthetize and

store biogenic monoamines (Falck *et al* 1964 Ritzen *et al* 1965 Larsson *et al* 1966, Cegrell 1968, Falck and Owman 1968 Tjalve and Slanina 1971) A direct effect of monoamines on the release of insulin and thyrocalcitonin has been described in various animal species (see Bates *et al* 1969 Philippo *et al* 1969 and Tjalve 1971) and a presence of intrinsic monoaminergic mechanisms operating in these cells has been discussed (Falck and Owman 1968 Pearse 1966, 1969, Ullberg and Hammarstrom 1969 Tjalve 1971, Melander *et al* 1971)

From the present results it seems that all the islet cells in the pancreas and parafollicular cells of the thyroid that are able to synthetize monoamines can also take up atropine An accumulation of atropine in the pituitary gland adrenal medulla and gastric mucosa that are also known to store monoamines (Falck and Owman 1968), has been described by Albanus *et al* (1968)

Since another common feature of these systems is the presence of pseudo- or true cholinesterase (Pearse 1966 1969 Carvalheira and Pearse 1967, Welsch *et al* 1967, Welsch and Pearse 1969), it is tempting to conceive that also acetylcholine might play some role in an intrinsic mechanism of polyepeptide hormone release and/or production

Parasympathetic agents have been shown to cause a stimulation of the insulin secretion an effect which has been shown to be blocked by atropine (Malaisse *et al* 1967 Kaneto *et al* 1968) It was proposed by these authors that this was an effect of a stimulation of the parasympathetic nervous system It also seems possible that it might in addition be a result of a stimulation of acetylcholinergic receptors present within the cells Since the actions of monoamines and acetylcholine in connection with neuronal functions are assumed to be based on the changes they induced in the permeability of membranes a similar function might be present also in extraneuronal tissues such as the pancreatic islets and the parafollicular cells (Tjalve 1971) A synergistic effect between several biogenic amines may be present within the cells (Ullberg and Hammarstrom 1969 Tjalve 1971)

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The Oxygen Supply to the Retina, I. Effects of Changes in Intraocular and Arterial Blood Pressures, and in Arterial P_{O_2} and P_{CO_2} on the Oxygen Tension in the Vitreous Body of the Cat

By

ALBERT ALM and ANDERS BILL

Received 18 August 1971

Abstract

ALM, A and A BILL *The oxygen supply to the retina, I Effects of changes in intraocular and arterial blood pressures, and in arterial P_{O_2} and P_{CO_2} on the oxygen tension in the vitreous body of the cat* Acta physiol scand 1972 84 261—274

The P_{O_2} in the vitreous body close to the retina P_{vrO} was determined continuously with an O_2 electrode In cats with a mean arterial blood pressure of 176 ± 7 cm H_2O a P_{aO_2} of 98 ± 2 mm Hg,

ough the retina is very efficiently autoregulated that oxygen tends to induce vasoconstriction in the retinal blood vessels, that hypocapnia gives vasoconstriction and hypercapnia marked vasodilatation

In cats as in primates the retina is nourished both by the blood vessels of the choroid and those of the retina Many of the factors that influence the blood flow through the choroid are now known There is an approximately linear relationship between the perfusion pressure, defined as the mean arterial blood pressure (MAP) the intraocular pressure (IOP), and the blood flow (Bill 1962a) The flow is strc influenced by electrical stimulation of the cervical sympathetic chain but only moderately influenced even by large alterations in the (Bill 1962c) The correlation that has been found between perfu blood flow and the rather poor sensitivity o CO_2 is surprising for volved in the nutrition of nervous tis In the brain the b influenced by changes in P_{aCO_2} and the is very eff

is the flow remains constant within a wide range of perfusion pressures (for a recent review of the regulation of cerebral blood flow see Häggendal, Nilsson and Norbäck 1969).

The inaccessibility of the retinal blood vessels precludes direct continuous measurements of the blood flow through the retina. Until now, therefore, knowledge about the regulation of retinal blood flow has been based mainly on inspection of the large vessels that can be observed in the fundus. Recently the labelled microsphere technique has been found useful for quantitative determinations of retinal blood flow (Alm and Bill 1972). One drawback of this method is that only one flow value can be obtained for each retina. This makes the method unsuitable for determinations of effects of graded changes in e.g. eye pressure or P_{aCO_2}.

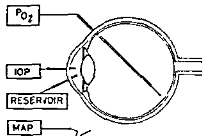
The purpose of the experiments reported here was to develop a method for continuous determination of the P_{O_2} in the vitreous body close to the retina. It was expected that such measurements should give useful information about factors that influence the P_{O_2} in the retina, including the blood flow through the retina. The reason for this belief is that the vitreous body has no blood vessels of its own and a very low O_2 consumption (Fischer von Bunau and Fischer 1932). Thus the P_{O_2} in the vitreous body is determined mainly by that in the surrounding tissues. Studies on the P_{O_2} at different places in the vitreous body of the rabbit eye have shown that the highest values prevail close to the retina and the lowest in the central parts behind the lens (Jacobi and Driest 1965). This means that O_2 diffuses from the retina towards the lens. Then one can expect that the P_{O_2} in the vitreous body close to the retina, P_{VrO_2}, depends mainly on the mean P_{O_2} in the innermost layer of the near by parts of the retina.

In this communication we wish to report on the influence of graded variations in IOP and MAP as well as in P_{aCO_2} and the arterial P_{O_2} (P_{aO_2}) on P_{VrO_2} in cats. The effects of large changes in IOP and P_{aCO_2} on the blood flow through the retina and the uvea as measured by labelled microspheres are reported in the following paper (Alm and Bill 1972).

Methods

A. *In vivo experiments*

Adult cats of both sexes weighing 1.9–5.4 kg were used. Anesthesia was induced with chloroform. A mixture of chloralose and pentobarbital sodium was then given i.v. (50–70 mg chloralose and 5—7 mg pentobarbital sodium per kg b.w.). Anesthesia was maintained by i.v. urethane not exceeding 100 mg/kg b.w./h. The femoral artery and vein on one side were cannulated, the vein for [illegible] nts of the air damped MAP with a [illegible] weden). The polyethylene tubings [illegible] had been added small amounts of [illegible] and artificially ventilated with a [illegible] as chosen to [illegible] bocurarin [illegible] The animal [illegible] ds tempera [illegible] ure was abo [illegible] lysed cal



in the lower nasal quadrant about 5 mm from the optic nerve head.

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was then connected both to the external reservoir and the pressure transducer In these experiments there was a *small pressure difference between the pressure transducer and the anterior chamber* when there was an inflow from the reservoir. The approximate value for this pressure fall at most ...

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The electrode is sensitive to changes in temperature The temperature coefficient was determined for each new membrane (see Beckman Technical Bulletin, PG TB-002) At the end of the experiments the temperature of the eye was measured with a thermocouple, introduced through the cannula used for the P_{O_2} measurements It was assumed that the difference between the eye and rectal temper...
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B *Model experiments*

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tip and the retina This possibility was tested in model experiments The equipment used is shown in Fig 2 The chamber above the teflon membrane was filled with either saline or vitreous body covered with mineral oil In the tube under the teflon membrane there was a steady flow of air

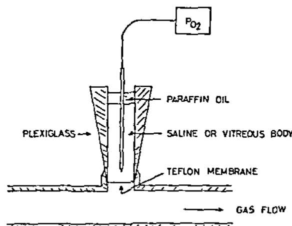


Fig 2 The equipment used in the model experiment

The electrode tip was placed very close to the membrane initially When a steady reading was obtained the gas was changed from air to 100 % O_2 The P_{O_2} recorded by the electrode increased and after some time reached a new steady level The time required to reach 10 % of the total rise, T_{10} was noted This procedure was repeated with the electrode tip withdrawn in steps of 0 5 mm

Results

A *Model experiments*

The results of the model experiments are shown in Fig 3 There was an approximately linear relationship between log T_{10} and the distance between the electrode and the membrane separating the gas from the fluid The results with saline and vitreous body were similar

B *In vivo experiments*

1 *Positioning of the electrode*

After the electrode had been inserted to a position judged to be 2—4 mm from the retina the animal was ventilated with 6 5 % CO_2 in O_2 for 3 min and the response of the electrode was observed The results of pilot experiments had indicated that

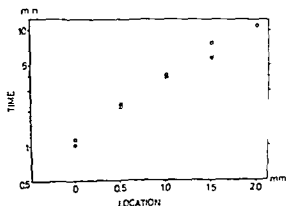


Fig 3 The time T_{10} plotted against the location of the electrode in the model experiment T_{10} is the time taken to reach 10 of the final rise in P recorded after a change from air to 100 % O_2 The point 0 on the location scale represent the position of the electrode very close to the membrane at the start of the experiment The electrode was then withdrawn in steps of 0 5 mm Open symbols represent saline closed symbols vitreous humor Note logarithmic time scale See text

this mixture always gives a rise in P_{VRO_2} of 25 mm Hg or more The time required for a clear rise (more than 5 mm Hg) in P_{VRO_2} was used to estimate the distance between the electrode tip and the retina If this response was observed within 5 min the position was accepted, otherwise the electrode was advanced to meet the "O_2 wave" The results of the *in vitro* experiments suggested that in most experiments, the electrode tip was within 2 mm from the retina At the end of the experiments after the animal had been killed by i v KCl, a sharp needle was inserted into the cannula that had supported the electrode The needle was pushed against the retina The eye was then removed and opened and the retina examined to localize the mark made by the needle and to ensure that the electrode had not damaged the retina Most measurements of P_{O_2} were made in the lower nasal quadrant 3—7 mm from the papilla

Several experiments had to be discarded since no steady P_{O_2} readings were obtained although the electrode functioned properly In most cases a satisfactory explanation was found when the eye was dissected, either a retinal detachment or a bleeding from the place of insertion or from a damaged retina In a few instances no such explanation could be found An air bubble may have been introduced into the vitreous body or the electrode may have touched the retina and produced a spasm in the retinal blood vessels

Sometimes several experimental changes could be made in the same animal However, certain experimental situations, especially prolonged hypoxia or hyperventilation apparently changed the normal vascular reactivity in the retina Thus after e g prolonged hyperventilation P_{VRO_2} did not respond to changes in the perfusion pressure or P_{aCO_2} in the same manner as before the hyperventilation None of the results described in the following were obtained after a previous experimental situation had changed the response of P_{VRO_2} to changes in perfusion pressure or P_{aCO_2}

2 *Starting data*

At the beginning of the experiments when the position of the electrode was accepted the MAP was 176±7 cm H_2O (n = 33) (here and throughout mean and S E are given), the IOP was stabilized at 32±2 cm H_2O (n = 33), the arterial P_{O_2} P_{CO_2} and pH were 98±2 mm Hg (n = 33), 27 0±1 2 mm Hg (n = 33 and 7 43±0 16 units (n = 24) respectively The P_{VRO_2} was 18 9±1 5 mm Hg n = 33

3 *Effects of changes in IOP on* P_{VRO_2}

The perfusion pressure for blood flow throug th a equal the blood pressure in the central retinal arteries minus the pressure ir t ai ins where they leave the retina The venous pressure can be assumed to be almost the same as the IOP at normal and high IOP The true arterial blood pressure on the other hand is not known It is lower than the MAP in the femoral artery For convenience the perfusion pressure in the experiments reported here was defined as the MAP in the femoral artery minus the IOP Fig 4 shows the relationship between the P_{VRO_2} and the perfusion pressure in 8 expts When this pressure was reduced by moderate artifi

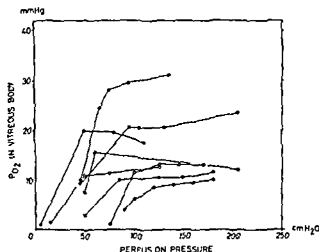


Fig 4 The effect on P_{vrO_2} of stepwise increments in intraocular pressure Perfusion pressure is defined as the mean arterial blood pressure in the femoral artery minus the intraocular pressure and expressed in cm H_2O Open symbols were used for experiments in which the actual intraocular pressure had to be calculated See text ($n = 8$)

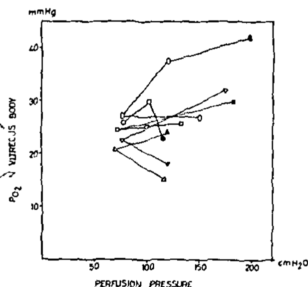


Fig 5 The effects on P_{vrO_2} of changes in perfusion pressure produced by changes in arterial blood pressure Closed symbols represent *starting values* ($n = 5$)

increments in IOP there was only little or no change in P_{vrO_2} *In most experiments,* however, at perfusion pressures between 100 and 50 cm H_2O further reductions resulted in marked decreases in P_{vrO_2}

4 *Effects of changes in MAP on* P_{vrO_2}

P_{vrO_2} was determined in 5 expts, in which the perfusion pressure was changed by bleeding *and retransfusion* MAP was *reduced from 120—240 cm* H_2O *to 70—115* cm H_2O and in most experiments brought back to normal values Fig 5 shows that in 3 expts P_{vrO_2} was almost unchanged or that it increased when the perfusion pressure was reduced *In 2 cases bleeding resulted in a fall in* P_{vrO_2} *but retransfusion then had*

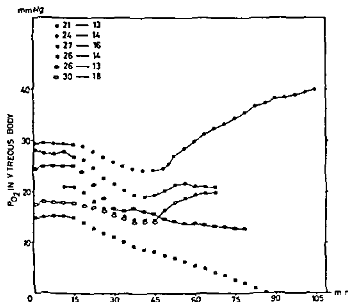


Fig 6 The effect of hyperventilation on P_{vrO_2}. The values for the arterial P_{CO_2} before and during hyperventilation are given in the upper left hand corner. Symbols not connected by lines represent period of hyperventilation (n = 6)

no effect on P_{vrO_2} or decreased it. Thus in the range of perfusion pressures investigated there was no unequivocal relation between the perfusion pressure and P_{vrO_2}

5 *Effects of changes in P_{aCO_2} on P_{vrO_2}*

In 6 expts hyperventilation of the animals to P_{aCO_2} values around 15 mm Hg consistently reduced P_{vrO_2}, Fig 6. After a period of hyperventilation P_{vrO_2} did not return to the initial level when the ventilation was normalized. In one case P_{vrO_2} remained at about zero for more than 30 min. In another it increased much above the original level and in the other experiments the results were varying.

When the animals were ventilated with 2—12 % CO_2 in air there was always a rise in P_{vrO_2} if the changes in P_{aCO_2} were produced at the beginning of an experiment. Fig 7 shows the result in 6 expts in which P_{CO} was changed from varying degrees of moderate hypocapnia (P_{aCO_2} below 25 mm Hg) to hypercapnia

6 *Effects of changes in P_{aO_2} on P_{vrO_2}*

In 5 expts P_{aO_2} was reduced in one or more steps, Fig 8. In 4 experiments each reduction in P_{aO_2} resulted in a reduction in P_{vrO_2}. The effect of increases in P_{aO_2} differed more between animals, Fig 9. In all 7 experiments were performed, in 2 of these the P_{vrO_2} was largely unchanged even at P_{aO_2} levels above 350 mm Hg, while in 2 experiments there were marked increases in P_{vrO_2}

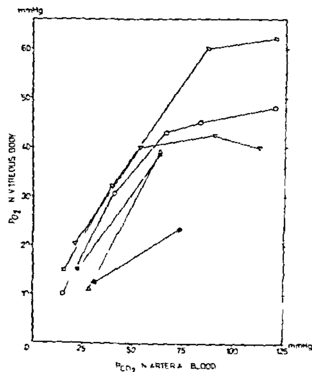


Fig 7 The effect of changes in arterial P_{CO_2} on P_{VTO_2}. The perfusion pressure changed during the experiments due to changes in arterial blood pressure. The perfusion pressure at the start and the end of the experiments were ■—■ 150 and 180 cm H_2O △—△ 130 and 190 cm H_2O ●—● 130 and 150 cm H_2O □—□ 85 and 185 cm H_2O ○—○ 125 and 110 cm H_2O ▽—▽ 180 and 140 cm H_2O (n = 6)

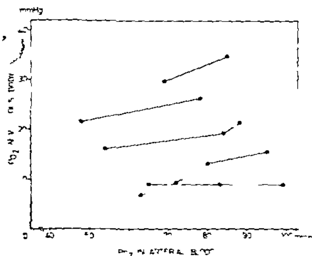
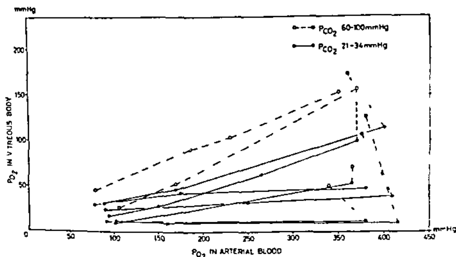


Fig 8 The effect on P_{VTO_2} of changes in arterial P_{O_2} (n = 5)

7 *Effects of high P_{aO_2} and high P_{aCO_2} levels on P_{VTO_2}*

In 6 of the experiments shown in Fig 9 where P_{aO_2} was increased to levels above 300 mm Hg the experiments were continued by ventilating the animals with 6.5 % CO_2 in O_2, which, in all instances resulted in an increase in P_{VTO_2}. In 2 experiments the effects of step-wise changes in P_{aO_2} on P_{VTO_2} were studied while P_{aCO_2} was kept above 75 mm Hg during the entire experiment. In these experiments each change in P_{aO_2} resulted in a marked change in P_{VTO_2} in the same direction Fig 9



Discussion

A Oxygen tension in the vitreous body

Measurements of P_{O_2} in tissues with O_2 electrodes similar to that used in the present experiments have been critizised mainly for two reasons, absolute values are not obtained if the calibration and the measurements are not made in the same or similar media, and the electrode tends to upset the local circulation and destroy the tissue in which it measures P_{O_2} (see Silver 1966) In the vitreous body these difficulties are largely overcome by the composition of the tissue There are no blood vessels in the vitreous humor and calibration in saline may be used since the P_{O_2} recorded in saline and vitreous body equilibrated with the same gas differed by less than 2 %

The values for the initial P_{VTO_2} averaged in "starting data" have been corrected for the difference in temperature between the calibration and measurements The mean value, 19 mm Hg is of the same order as that reported for the rabbit eye (Jacobi and Driest 1965) It is considerably lower than the value reported by Krause and Goren (1956) for the cat eye, 53 mm Hg These investigators made their measurements at an unspecified place in the vitreous body after removal of the cornea and the lens and at an eye pressure of zero No doubt these factors explain the discrepancy The P_{VTO_2} values shown in the figures in this study are not absolute values since no corrections have been made for slight electrode drift and small changes in eye temperature during the experiments Both these processes, however result in small and slow changes in electrode current compared to the changes produced by changes in retinal oxygen tension

B Oxygen tension in the retina

The findings of Jacobi and Driest (1965) that the P_{O_2} is higher close to the retina than in the central parts of the vitreous body were confirmed in this study During insertion of the electrode the recorded P_{O_2} increased as the electrode tip came closer to the retina The increase usually was 1/2—2 mm Hg per mm This means that the true mean P_{O_2} in the part of the retina studied can be expected to be a few mm Hg above the measured P_{vrO_2} In the brain of the dog the mean P_{O_2} on the surface is 16 mm Hg (Jones, Crowell and Smith 1969) while that in venous blood is about 25 mm Hg (Davies and Bronk 1957) A similar difference seems reasonable for the cat retina This assumption suggests a retinal venous P_{O_2} of 30—35 mm Hg in the cat, corresponding to an O_2 saturation of 40—45 % (Bartels and Harms 1959). This would correspond to an O_2 extraction from retinal blood of 8—9 vol % In man Hickam, Sieker and Frayser (1959) reported a retinal venous O_2 saturation of 57±11 % (M±S D) Compared to these figures the O_2 saturation in venous blood leaving the choroid in the cat is very high, about 90 % (Alm and Bill 1970)

When a change in the P_{O_2} of the retina was produced truly steady P_{vrO_2} was not always attained which was due to the time needed for equilibration of the whole vitreous body An acceptable steady state was considered to be established when the change in P_{vrO_2} was less than 5 % in 5 min Thus all changes in retinal P_{O_2} tended to be underestimated quantitatively In experimental situations where the P_{aO_2} and retinal O_2 consumption are unchanged a steady P_{vrO_2} indicates a steady retinal blood flow Changes in retinal blood flow can be expected to produce qualitatively similar changes in P_{vrO_2}

C Effects of changes in IOP or MAP on P_{vrO_2}

Autoregulation of cerebral blood flow was suggested by Fog (1934) who used the pial window technique Later investigations with many different techniques for blood flow determination have fully confirmed that cerebral blood flow is efficiently autoregulated Porsaa (1941) seems to have been first to report evidence for autoregulation of the blood flow in the retina He used a technique that was similar to that used by Fog The inaccessibility of the retinal blood vessels has, until now precluded verifications of the results of Porsaa with methods others than those based on inspection of the fundus

In the present experiments P_{vrO_2} was largely unchanged during moderate reductions in perfusion pressure when these were produced by changes in IOP When they were produced by changes in MAP the response was varying but there was no correlation between perfusion pressure and P_{vrO_2} As a rule a marked reduction in P_{vrO_2} was not seen until the perfusion pressure was reduced to values between 50 and 100 cm H_2O These results indicate that the blood flow through the retina is not reduced by a moderate reduction in perfusion pressure Quantitative measurements of retinal blood flow at high IOP indicate that there may even be a rise in blood flow (Alm and Bill 1972)

In the brain the blood flow is largely independent of the perfusion pressure at levels above 45—60 cm H_2O (see Haggendal, Nilsson and Norback 1969) In the experiments reported here the true perfusion pressure is somewhat overestimated as mentioned above Thus the blood flow through the retina seems to be autoregulated within the same range of true perfusion pressures as that through the brain

The observation that the P_{VTO_2} was less stable after a reduction in perfusion pressure caused by changes in MAP than when caused by changes in IOP may be explained by changes in the composition of the blood In cats bleeding may either increase or decrease the hematocrit (Mott 1968) Thus, the changes in P_{VTO_2} observed may have been caused by changes in hematocrit and not by changes in blood flow

D *Effects of changes in P_{aO_2} on P_{VTO_2}*

Hypoxia has been reported to dilate the arterioles in the retina (Hickam and Frayser 1966) and the pial vessels in the brain (Wolff and Lennox 1930) In the brain recent investigations have shown that no appreciable increase in blood flow appears until the P_{aO_2} is reduced to levels of about 50 mm Hg (see Reivich 1964)

In this study reductions in P_{aO_2} resulted in clear reductions in P_{VTO_2} in 4 experiments of 5 The mean fall in P_{VTO_2} was 1 5—2 0 mm Hg per 10 mmHg fall in P_{aO_2} In cats a reduction in P_{aO_2} from 90 to 60 mmHg results in a reduction of the O_2 content of about 2 vol % If we assume that the O_2 saturation of retinal venous blood is about 40 % (see above) a similar reduction in O_2 content would result in a fall in P_{O_2} of 7 mmHg which is slightly more than the observed average fall in P_{VTO_2} In the choroid venous blood is normally saturated to about 90 % and a reduction of the perfusion pressure to half the original value results in a fall in P_{O_2} of 10—15 mmHg (Alm and Bill 1970) This fall in choroidal venous P_{O_2} is of the same order as that expected if P_{aO_2} is reduced from 90 to 60 mmHg A reduction of the perfusion pressure to half the original value did not reduce P_{VTO_2} which was due to vasodilatation in the retina It can be calculated that also in the experiments with hypoxia a fall in P_{VTO_2} would have been prevented by an increase in blood flow through the retina of less than 50 % The vasodilatation that may have occurred in the present experiments with hypoxia in most cases was not enough to prevent a fall in P_{VTO_2} Thus it is unlikely that autoregulation of retinal blood flow is based on high P_{O_2} sensitivity of the vascular smooth muscle or of some kind of receptors

O_2 at atmospheric pressure causes constriction both of pial vessels in the cat (Wolff and Lennox 1930) and retinal vessels in man (Hickam and Frayser 1966) and in kittens (Ashton 1968) Kety and Schmidt (1948) reported a reduction in cerebral blood flow in man breathing pure O_2 a result not verified in later investigation in monkeys (Reivich 1964)

In the part of the retina supplied by the choroidal blood vessels a rise in P_{aO_2} can be expected to raise the P_{O_2} since 100 % O_2 has no effect on the vascular resistance in the uvea (Bill 1962c) and a rise in P_{O_2} in the choriocapillaris will increase the P_{O_2} in the retina (Dollery, Bulpitt and Kohner 1969) In this study the effects of increases in P_{aO_2} on P_{VTO_2} were varying In experiments where there was no change

in P_{VTO_2} there can be little doubt that O_2 produced a marked fall in retinal blood flow In some experiments, however, the vasoconstriction — if present — was inadequate to keep the tissue P_{O_2} constant Hickam, Sieker and Frayser (1959) also found an increased O_2 saturation in retinal venous blood in humans breathing 100 % O_2

E *Effects of hypo- and hypercapnia on P_{VTO_2}*

The vasoconstrictive effect of hypocapnia and the vasodilative effect of increased P_{aCO_2} in the brain are firmly established Pial arteries in cats respond to changes in P_{aCO_2} (Wolf and Lennox 1930) and the blood flow through the brain is increased both in man (Kety and Schmidt 1948) and monkeys (Reivich 1964) breathing CO_2 in air Huerkamp and Rittinghaus (1950) reported inconsistent changes in the diameter of the retinal vessels in man during hyperventilation In our study hyperventilation produced a variable fall in P_{VTO_2} suggesting a reduction in retinal blood flow In one case P_{VTO_2} fell to zero and did not return when ventilation was normalized which suggests that the hyperventilation gave a persistent spasm of the retinal blood vessels Lende and Ellis (1964) have reported that mechanical stimulation of the retinal blood vessels may produce a spasm

Hypercapnia has been reported to cause retinal vasodilatation in dogs (Spalter TenEick and Nahas 1964) In man Huerkamp and Rittinghaus (1950) reported dilatation but other investigators have failed to verify this result (Hickam and Frayser 1966 A decrease in retinal a v O_2 difference caused by breathing 10 % CO_2 in air Frayser and Hickam (1964) to assume that an increase in blood flow had taken place caused by dilatation of vessels too small to be studied on fundus photographs Bulpitt Dollery and Kohner 1970) have recently reported that during hypercapnia in pigs there is a widening of the peripheral plasma zone in the retinal arteries which complicates measurements of the vascular diameter

In the present study P_{VTO_2} increased markedly when the P_{aCO_2} was increased to values around 75—100 mmHg This strongly suggests that CO_2 has a marked vasodilative effect on the retinal blood vessels in cats However it is possible that at very high levels of P_{aCO_2} there was a reduction in retinal O_2 consumption that contributed to the high P_{VTO_2} observed In vitro experiments have shown that the metabolism of the retina is disturbed at increased P_{CO_2} which results in decreased O_2 consumption (Craig and Beecher 1943 It is also likely that the Bohr effect of CO_2 on the dissociation of oxyhemoglobin somewhat contributed to the changes observed in P_{VTO_2} However, experiments with quantitative determinations of retinal blood flow confirm that CO_2 has a marked vasodilatory effect in the retina (Alm and Bill 1972

F *Oxygen supply to the retina at increased P_{aO_2} and P_{aCO_2}*

Mathematical calculations have led Dollery Bulpitt and Kohner (1969) to assume that about 60 % of the avascular zone of the retina is supplied with O_2 from the choroid at normal P_{aO_2} and that this figure would increase to about 97 % at a P_{aO_2} of 400 mmHg In the present experiments a P_{aO_2} of 350 mmHg combined with high P_{aCO_2} levels resulted in P_{VTO_2} values of 100—150 mmHg This means that the O_2

extraction from retinal blood was reduced from 8—9 vol % to about 1 vol % This is partly caused by an increase in blood flow, since high levels of P_{aCO_2} increase the blood flow through the retina 3—4 times, but the O_2 extraction per min from the retinal vessels will still be less than half of that observed at normal blood gas tensions Thus, it is likely that at high levels of P_{aO_2} and P_{aCO_2} the choroidal blood vessels supplied O_2 to a larger part of the retina than under normal conditions

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The Distribution of Xe-133 in the Left and Right Ventricular Walls of Dog Hearts after Left Ventricular Bolus Injection

By

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Abstract

BAGGER, H *The distribution of Xe 133 in the left and right ventricular walls of dog hearts after left ventricular bolus injection* Acta physiol scand 1972 *84* 275—283

Regional coronary blood flow was estimated in dogs by counting the activity in tissue blocks of the left and right ventricular walls after bolus injection of Xe-133 into the left ventricular cavity

Myocardial uptake of the isotope was 1 7 times higher in the left than in the right ventricular wall, while comparison of the activities in the subepicardial half of the walls showed a ratio left/right of 1 5

The transport through the endocardial surface of the left ventricular wall was measured during constriction of the left coronary artery Xe 133 penetrates to a depth of 7 mm indicating that transport across the endocardial surface might contribute to the activity of the subendocardial half of the wall

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[illegible] and circumflex branches of the left coronary artery or between the apex and the base

In the right ventricular wall there was no difference between subepi and subendocardial activity

Estimation of local myocardial blood flow from the disappe[illegible] of intra myocardial desposits of NaJ 131 (Kirk and Honig 1964) and [illegible] (Ha[illegible] Harper and Estes Jr 1966 Brandi Fam and McGregor 1968 Andersen Bagger and Gotzsche 1969) have shown that tissue blood flow in the superficial layers of the left ventricular wall is 25—30 % higher than in the deep layers However it has been argued (Moir and DeBra 1967) that tissue isotope clearance is influenced by local edema and hemorrhage from the injection which together with leakage of isotope up the needle track might cause the difference in disappearance rate

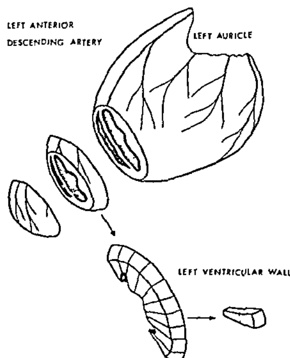


Fig 1 Slicing of the heart

In the study presented in this report the distribution of Xe-133 was measured by counting the activity in tissue blocks of the right and left ventricular walls of dog hearts after bolus injection of the isotope into the left ventricular cavity Assuming that the isotope is well mixed with blood at the aortic root, that xenon is delivered to the tissue only by the coronary arteries, that transport of xenon from blood to tissue is taking place only at capillary level and that the isotope remains in the tissue to which it is transported by blood, during the preparation of the heart, the activity per unit weight is proportionate to the flow, and differences in flow to various areas of the myocardium can be estimated

Methods

Mongrel dogs weighing 21—55 kg were used for the experiments Thiomebumal sodium (Leopental®) was given i v (15—20 mg per kg) and ventilation maintained with atmoshperic air through an endotracheal tube with an Engstrom ventilator

A Ducor ventricular "pig tail" catheter was introduced through the right femoral artery and under fluoroscopy the tip was placed in the bottom of the ventricular cavity A polyethylene catheter (outer diameter 1 5 mm) was introduced through the left femoral artery to allow continuous observation of aortic pressure together with simultaneous recording of the ECG Thoracotomy was performed on the left side and the pericardium was opened During the experiments repeated measurements of oxygen saturation, oxygen and carbon dioxide tensions, pH and standard bicarbonate in arterial blood were performed as further controls of normal circulation and ventilation

Xe 133 (The Radiochemical Centre, Amersham England) was injected as a bolus into the left ventricular cavity and the heart was immediately excised

TABLE I Mean activities of left and right ventricular walls of 5 dogs after injection of Xe 133 in the left ventricular cavity

Dog no	Activity injected mCi	Number of tissue blocks		Mean activity cps/100 mg ± SE		p	Left/right
		Left	Right	Left	Right		
74	2 4	19	12	195±4	108±5	<0 001	1 80
75	2 4	16	12	176±4	120±3	<0 001	1 46
76	1 9	15	17	101±4	53±2	<0 001	1 90
78	3 0	17	11	235±6	149±3	<0 001	1 57
79	3 0	17	15	159±3	100±4	<0 001	1 59

0 6—7 7 mCi Xe 133 was injected into the left ventricular cavity in 16 dogs Injection time was 0 7—3 0 s The heart was removed in 10—26 s after the injection.

In 4 dogs the main trunk of the left coronary artery was isolated by blunt dissection and a loose ligature was placed around the artery An interval of 45 min was interposed after which the artery was constricted and 0 5—2 7 mCi Xe 133 injected into the left ventricular cavity Injection time was 1 4—2 6 s The heart was removed in 8—14 s after the injection

Following removal of the heart the ventricular cavities were flushed first with isotonic saline at room temperature and second with iso-pentane cooled with dry ice to −75° C The heart was then transferred to a 6 l Dewar flask filled with a mixture of dry ice and iso-pentane (−75° C) ... from the ape[illegible] of the heart [illegible] and divided i[illegible] and placed in previously weighed cooled closed tubes Slicing of the heart was performed on dry ice with further cooling in the mixture of dry ice and iso-pentane every 5—10 s

The activity was counted at the photopeak of xenon at 81 keV in a well type scintillation detector, after which the tubes were weighed and the activity expressed as cps/100 mg tissue

The possibility of diffusion of xenon from the tissue before and during freezing of the heart was estimated b[illegible] 2[illegible] o[illegible] a[illegible] tissue the area of activity was recorded for periods of 10 s by a gamma camera (Pho/gamma 3 Nuclear Chicago) During 15 min the area of activity was unchanged The possibility of diffusion of xenon from the tissue after freezing was estimated by tranferring every other tissue block of a slice to the counting tubes immediately after slicing and letting the others remain in the mixture of dry ice and iso-pentane until the rest of the heart had been sliced The activity was not different in the two groups

Results

In 5 of the experiments with free left coronary artery flow the activity was measured in both left and right ventricular walls The activity in the left ventricular wall was significantly higher than that in the right ventricular wall (Table I However the activity in the left ventricular wall might partly be due to uptake of xenon from the ventricular cavity or blood containing xenon on the endocardial surface Therefore on three dogs Xe 133 was injected immediately after constriction of the left coronary artery In none of the dogs were changes observed in blood pressure ECG or heart rate, during the injection The results (Table II) show that there was very little activity in the epicardial half of the left ventricular wall, while significant activity was found in the endocardial half To localize the xenon in the endocardial area

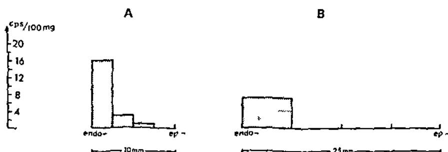


Fig 2 Myocardial activity of a tissue block between the anterior and posterior papillary muscle (A) and a tissue block containing the posterior papillary muscle (B), after injection of Xe 133 into the left ventricular cavity during constriction of left coronary artery

each tissue block from the left ventricular wall of a fourth dog was split into an endo and epicardial half, each of which was further divided in half In Fig 2 A represents the activity in a tissue block 10 mm in thickness, from the area between the anterior and posterior papillary muscle, B the activity in a block 25 mm in thickness containing the posterior papillary muscle In both blocks xenon was recorded at a distance of 6—8 mm from the endocardial surface

The difference in activity between the left and right ventricular walls can partly be explained by uptake of xenon from the left ventricular cavity As this uptake contributes mainly to the activity of the endocardial part of the wall, an expression of the activity delivered to the left and right ventricular walls by the coronary arteries can be obtained by comparing the activity of the epicardial halves of the walls (Table II)

A representative result of the distribution of Xe-133 in the epi and endocardial halves of the left ventricular wall is shown in Fig 3, the activities of the tissue blocks of each slice are plotted in the direction from the left anterior descending branch towards the circumflex branch and the slices numbered in the direction from the apex towards the base of the heart The activity level is constant from slice to slice and constant in the tissue blocks of each slice independent of blood supply from the anterior descending or circumflex branch of the left coronary artery Fig 4 shows a comparison between the mean epi and endocardial activity of the left

Table II Mean activities of epi and endocardial halves of left ventricular wall of 3 dogs during constriction of left coronary artery

Dog no	Activity injected mCi	Number of tissue blocks	Epicardial half cps/100 mg ± SE	Endocardial half cps/100 mg ± SE
53	0 6	30	1 ± 0 2	9 ± 1 3
73	2 4	17	1 ± 0 2	34 ± 7 0
77	2 7	16	1 ± 0 2	45 ± 6 2

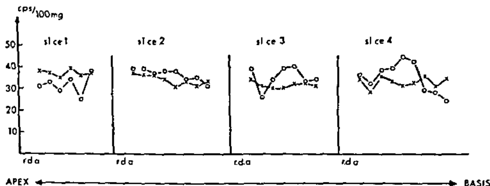


Fig 3 A representative result of the distribution of Xe 133 in the epi and endocardial halves of 4 slices of the left ventricular wall The activity of the blocks of each slice is shown in the direction from the anterior descending towards the circumflex branch of the left coronary artery

ventricular wall of 12 dogs, each point represents a mean value of about 25 tissue blocks. The slope of the regression line, b_{yx} is 0 85 ± 0 03, which is significantly different from 1 ($p < 0\ 005$), the correlation coefficient 0 99 and the intercept (y,0) 6 5

The distribution of xenon in the epi- and endocardial halves of the right ventricular wall of 9 dogs is shown in Fig 5, plotted in the same way as in Fig 4 The slope of the regression line, b_{yx} is 0 82 ± 0 14, which is not significantly different from 1 ($p < 0\ 30$), the correlation coefficient 0 91 and the intercept (y, 0) 10 8

Discussion

The ratio of activity for the left and right ventricular walls of approximately 1 5 confirms the results obtained after injection of radioactive microspheres into the left atrium (Domenech *et al* 1969) They also found a good correlation between coronary blood flow calculated from the myocardial activity and from the venous

TABLE III Mean activities of epicardial region of left and right ventricular walls of 5 dogs

Dog no	Mean activity of epicardial half cps/100 mg ± SE		p	Left/Right
	Left	Right		
74	185 ± 4	128 ± 8	<0 001	1 44
75	172 ± 4	122 ± 5	<0 001	1 40
76	99 ± 4	61 ± 3	<0 001	1 62
78	220 ± 6	151 ± 4	<0 001	1 45
79	150 ± 2	95 ± 6	<0 001	1 58

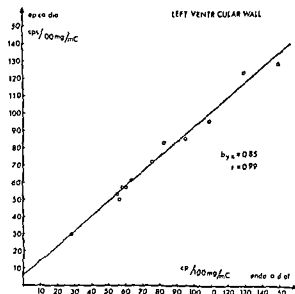


Fig 4

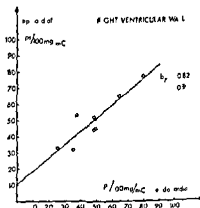


Fig 5

Fig 4 Comparison of mean epi and endocardial activity (cps/100 mg/mCi injected) of the left ventricular wall of 12 dogs

Fig 5 Comparison of mean epi and endocardial activity (cps/100 mg/mCi injected) of the right ventricular wall of 9 dogs

return in the coronary sinus This was interpreted as proof of adequate mixing of microspheres and blood at the aortic root The good correlation of the xenon distribution with the results obtained with microspheres indicate that myocardial uptake of Xe 133 is proportionate to flow The 50 % higher activity in the left than right ventricular wall confirms results obtained with measurements of myocardial uptake of Rb-86 after continuous i v infusion (Love and Burch 1957) and 20 s and 10 min after rapid i v injection (Levy and Oliveira 1961) Furthermore Pitt Friesinger and Ross (1969) measured flow rates from wash out curves after injection of Xe 133 into the left and right coronary arteries and found a ratio left/right of 2 15 Aukland *et al* (1967) used hydrogen polarography and obtained a ratio of 1 12 As the total *pressure drop in the vascular system of the right ventricular wall is equal* to that of the left the difference in flow to the two ventricular walls must indicate that the mean resistance of the vascular system is higher in the right than left ventricular wall

In the left ventricular wall no differences were observed in uptake of xenon between the apex and the base of the heart or between areas supplied by the left anterior descending and circumflex branches A 15 % higher activity was recorded in the endo- than epicardial half of the wall This difference is significant and is of the same order as that found by Love and Burch (1957) Moir and DeBra (1965) and Gillespie and Love (1967) after i v infusion of Rb 86 However, Moir and

DeBra (1965) have shown that there is a direct uptake of Rb-86 from the left ventricular cavity after i v infusion Furthermore Myers and Honig (1966) detected the same isotope after i v infusion at a distance of 7.5 and 17 mm from the endocardial surface in areas of the left ventricular wall without and with papillary muscle respectively My results show that xenon also is able to cross the endocardial surface However, the experiments were performed during constriction of the left coronary artery and since collateral flow to the ischemic area cannot be excluded the results of Table II give the maximum contribution by diffusion. Injection of radioactive microspheres during constriction of the circumflex branch of the left coronary artery (Becker *et al* 1971) showed lower collateral flow to the endo- than epicardial region of the ischemic area If these results are applied to my experiments, the very low epicardial activity indicates that collateral flow to the endocardial region must be negligible Thus, comparing the results obtained with free flow with those during constriction of the left coronary artery, there seems to be no difference in amounts of activity transported by the coronary arteries to the endo- and epicardial regions of the left ventricular wall

In order to consider the uptake of xenon in different regions of the heart as being proportionate to flows, it was assumed that transport of the isotope from blood to tissue is taking place only at capillary level As the endocardial layer of the myocardium is supplied via arteries passing through the epicardial region a loss of activity through the arterial walls cannot be excluded Assuming this leakage, one might expect a perivascular activity higher than that of the surrounding heart muscle in the superficial part of the wall However, macro-autoradiography of the myocardium immediately after injection of Xe-133 into the coronary arteries (Shaw *et al* 1966) showed a uniform distribution of the isotope in the tissue

Injection of microspheres with a mean diameter of 14 μm (Domenech *et al* 1969) showed a ratio between deep and superficial activity of the left ventricular wall of 1 28 Becker *et al* (1971) using 15 μm microspheres found a ratio deep/superficial of 1 04—1 17 As stated by Domenech *et al* (1969) the prerequisite for interpreting the ratio of radioactivity in two different regions as a ratio of flows is, that the amount of activity in a layer is proportionate to the number of microspheres in it This cannot be assumed because the microspheres of a given mean diameter vary in volume and so in activity Therefore it must be assumed that ratios of small to large microspheres in the deep and superficial regions are of equal quantity, In dog hearts Provenza and Scherlis (1959) found that the most frequent type of branching after invasion of the myocardium by the superficial arteries is one in which the daughter vessels are of disproportionate diameter The larger of these vessels continues to progress through the myocardium slowly decreasing its diameter, while the smaller vessel is rapidly reduced in diameter terminating in a capillary network in the immediate area of bifurcation These observations correspond to the classification of A' and B vessels to the superfical and deep regions of the left ventricular wall of human hearts (Estes *et al* 1966) As the mean diameter of even the smallest microspheres used in the quoted reports is significantly higher tha

that of erythrocytes this variation in arterial size might lead to different ratios of small to large microspheres in the deep and superficial regions thus erroneously indicating different flows to the regions Here it must be mentioned that injections of microspheres of 20—22 μm and 50—60 μm (Domenech *et al* 1969) increased the ratio deep/superficial to 1 43 and 2 25 respectively Also plasma skimming might lead to higher activity in the deep than superficial region in the experiments using microspheres but this possibility is hardly probable as Myers and Honig (1964) found that tissue hematocrit values of deep and superficial regions are not different

In summary technical errors in the measurements of regional myocardial uptake of Rb 86 radioactive microspheres and Xe 133 result in a slight overestimation of flow to the endo in relation to the epicardial region of the left ventricular wall but for different reasons However considering these errors the blood flow seems to be uniform in the left ventricular wall of normal dog hearts

According to several authors (Ross *et al* 1964 Klein Cohen and Gorlin 1965 Bassingthwaighte Strandell and Donald 1968 Pitt *et al* 1969, Andersen *et al* 1969) the wash out curve recorded after injection of Xe 133 into the left coronary artery is not mono exponential as might be expected if the left ventricular wall is uniformly perfused By macro autoradiography immediately after injection of Xe 133 into the left coronary artery Shaw *et al* (1966) demonstrated a uniform activity in the myocardial wall Later when the wash out had deviated from a mono exponential function they found little radioactivity in the myocardium but a relatively large concentration in epicardial fat In accordance with these results the author (unpublished) found that 3 min after injection of Xe 133 into the left ventricular cavity macro autoradiography showed uniform activity in the muscle tissue but significantly higher activity in the perivascular tissue especially surrounding the larger epicardial vessels Thus the deviation from a mono exponential function might be explained by a slowly increasing activity in the perivascular fat and connective tissue as a result of diffusion through the very thin wall of the veins (Provenza and Scherlis 1959) followed by a slow wash out from this tissue due to the marked affinity of fat for xenon and the low perfusion of fat tissue This model of wash out with two compartments in series also seems able to explain why better correlation is obtained when flowmeter measurements of myocardial blood flow are compared to flow calculated from the initial wash out of Xe 133 after subtraction of pre clearance background than to flow calculated from the initial wash out after terminal slope subtraction (Pitt *et al* 1969)

In a previous report (Andersen *et al* 1969) the wash out after injection of Xe 133 into the left coronary artery was conceived as a sum of wash out curves from two or more compartments in parallel This model which is invalidated by the present results was assumed because blood flows calculated from wash out curves obtained after local injections into the left ventricular wall were 30 % higher in the epi than endocardial area The cause of this difference is not known but again diffusion phenomena might intervene retarding slightly the xenon clearance from the deep tissue

In the right ventricular wall to which xenon is transported exclusively by coronary blood no significant difference was found between activities of the deep and superficial regions. Accordingly Gillespie and Love (1967) only demonstrated higher activity in the deep than superficial part of the wall, if the isotope had been present in the right ventricular cavity.

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Acta physiol. scand. 1972. 84. 284—288

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Method for Studying Qualitative and Quantitative Changes of Blood Flow in Skeletal Muscle

By

PER-INGVAR BRÅNEMARK and ELOF ERIKSSON

Received 1 September 1971

Abstract

BRÅNEMARK P-I and E ERIKSSON *Method for studying qualitative and quantitative changes of blood flow in skeletal muscle* Acta physiol scand 1972 *84* 284—288

A vital microscopic method for studying changes of blood flow in skeletal muscle has been [illegible]

The circulation in skeletal muscles has been studied mainly with methods based on recordings of the blood pressure, the regional blood flow and plethysmographic changes The development of the methods can be traced from Gaskell (1877) to *e g* Folkow (1955) and Mellander (1960) These methods have provided quantitative data on the circulation in the musculature of entire limbs or parts of them Careful analysis of the data thus obtained has allowed conclusions on the sequence of events from a qualitative point of view But it has proved difficult to analyse the factors regulating flow distribution within the tissue from data obtained by these indirect measuring methods

The first intravital studies of the microcirculation in skeletal muscles were reported by Rous *et al* in 1930 He used the sartorius muscle of the frog and various thin muscles of mice, guineapigs, rabbits and cats Algire (1954) studied the circulation in the musculature of the back of the rabbit with the chamber technique Zweifach and Metz (1955) used the trapezius muscle of the rat This method has later on been modified and used by Hyman and Paldino (1962) and Gray (1971) Illig (1961) gives a good review of the methods used

Preparation of skeletal muscle for vital microscopy is technically difficult The



Fig 1 Muscle preparation *in situ* ready for intravital analysis

muscle layer is often not thin enough to allow transillumination with ordinary microscopic optics In addition, it is difficult to dissect skeletal musculature for microscopic studies without disturbing the local nervous and vascular function Immobilization of the animal is also problematic (see Brånemark 1959). Owing to these difficulties it has not been possible to study the blood cells or the vascular wall with good resolution Certain observations made by Zweifach and Metz (1955), for example, in their study of the precapillary sphincters in skeletal musculature have, however, proved useful in the analysis of the local flow distribution

Methods

On the basis of a suggestion put forward by dr Ralph Sonnenschein, UCLA Los Angeles, we [illegible]

Th [illegible]

[illegible]

[illegible] a rule, the nerves and vessels supplying the muscle enter its medial part with the result that they are not damaged at the preparation of the muscle The muscle should be dissected as carefully and gently as possible because even minor haemorrhage in the muscle is sufficient to preclude microscopic examina[illegible]

[illegible]

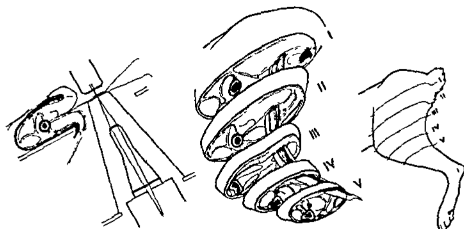


Fig 2 Schematic representation of the rabbit leg and optical set up for transillumination of skeletal muscle *in situ* The observed area is at the level of section II

By

...rded by photography with a film ...ope (Brånemark 1959) The corpuscular flow rate is recorded with a ...ot method (Brånemark and Jonsson 1963) The blood pressure, pulse rate and pulse pressure are recorded through a catheter placed in the subclavian artery and connected to 2 writers via a transducer (Statham P23 AC) The venous return from the left hind leg is registered with an optical drop recorder connected to the left femoral vein To allow stimulation of the ipsilateral sympathetic chain its lumbar portion is dissected and placed in a bipolar ring electrode

Discussion

Pilot expts showed that the muscle preparations are fairly stable and can be examined for up to 8 h without signs of microvascular injury The entire vascular tree as well as single vessels segments can be studied (Fig 3—4) The preparation also allows observation of the muscle during work (isometric contraction) To secure exact definition of the muscle specimens for histological and electronmicroscopic examination are obtained at every exp With these specimens it is possible to study also *e g* the myoglobin and hemoglobin content of the muscle To enable numerical calculation of the blood supply it is intended to determine the diameters of the muscle fibres as well as the diameters and lengths of all vessel segments In order to control the entire circulatory situation simultaneous recordings were made of a number of variables (blood pressure pulse pulse pressure venous return and ventilation status) These measurements together with determinations of the flow rate in different vessel segments should allow a good estimation of the blod flow through the resting muscle

As soon as the circulation in the resting muscle has been explored in detail it is intended to study the circulation under various physiological and pharmacological



Fig 3



Fig 4

Fig 3 Microphotogram of nutritive capillary of skeletal muscle in cat showing a discrete endothelial lining and characteristic deformation of the red cells The striation of the muscle fibres is visualized in detail

Fig 4 a b c 3 microphotograms of the capillary in Fig 3 illustrating the dynamics of blood flow in skeletal muscle capillaries of the cat recorded with 2 s intervals

conditions It is thus planned to study the circulation during exercise and during hemorrhagic shock The preparation lends itself well for testing of the effect of various drugs on the circulation in skeletal muscle For this purpose it is of course, necessary to study the change in the microcirculation and general circulation simultaneously

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Acta physiol. scand 1972 84 289—294
From the Department of Physiology, Karolinska Institutet, Stockholm Sweden

Light Sensitivity of the Compound Eye of a Moth (Manduca Sexta) Reared on a Retinol Deficient Diet

By

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Abstract

BOETHIUS, J, S D CARLSON, G HOGLUND and G STRUWE *Light sensitivity of the compound eye of a moth (Manduca sexta) reared on a retinol deficient diet* Acta physiol scand 1972 *84* 289—294

The photoreceptor response was recorded in the compound eye of a moth (*Manduca sexta*) reared on a retinol (vitamin-A) deficient diet The configuration, amplitude and spectral sensitivity of the receptor potential was similar to that found for normal eyes. The retinol deficient eyes are structurally deranged The present results therefore indicate that structurally deranged photoreceptors response generate normal receptor potentials

Retinol (vitamin A) deficiency causes a decrease in photosensitivity both in vertebrates (Dowling and Wald 1958) and insects (Goldsmith Barker and Cohen 1964) probably due to insufficient synthesis of photopigment In addition there is a structural derangement of the retina in rats (Dowling and Wald 1958), moths (Carlson *et al* 1967, Carlson, Gemne and Robbins 1969) and mosquitoes (Brammer and White 1969)

Flies deficient in retinol do not react to light and their electrophysiologically tested photosensitivity is decreased (Goldsmith Barker and Cohen 1964 Goldsmith and Fernandez 1966) A reduced response to light is also observed in retinal deficient mosquito eyes (Brammer and White 1969) In the retinol deficient moth *Manduca sexta* (Carlson, Gemne and Robbins 1969) the retinular microvilli are misaligned and the mitochondria dislocated from their normal position close to the rhabdomeres to a position on the ommatidial periphery

The present study concerns the possible influence of retinol deficiency on the light sensitivity in the compound eye of moths

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Fig 1



Fig 2

Fig 1 Response evoked by light stimulation of photoreceptor layer in compound eye from moth reared on retinol deficient diet Optic lobes removed Stimulus (200 ms) indicated by horizontal bar Vertical bar 1 mV Negativity upwards

Fig 2 Electroretinogram from moth reared on retinol deficient diet Note potential changes generated in optic lobes (positive deflection at beginning and negative deflection at end of stimulation) Stimulus (1 s) indicated by horizontal bar Vertical bar 2 mV Negativity upwards

Methods

The moths (*Manduca sexta* Lepidoptera Sphingidae) were reared for over 20 generations under septic conditions on a diet (Hoffman Lawson and Yamamoto 1966) deficient in retinol or its precursors The diet possibly contained trace amounts of β carotene (less than 3 μ per kg) and retinol (less than 6 μ per kg) The normal tobacco leaf diet contained 60 mg β carotene per kg and less than 1600 μg retino

The eye was stimulated by light (duration

This radiation passed consecutively through

neutral density wedge (Kodak) and neutral density filters (Bausch & Lomb) Relative photon flux was measured by a photomultiplier tube (RCA 1 P 28) maintained at a constant voltage by a high voltage supply (Oltronix)

The moth was dark adapted for 1 h before recording The head was severed from the body and put on a filter paper moistened with saline (Ephrussi and Beadle 1936) Extracellular records were obtained by the use of an electrode (Ag AgCl diameter 0 1 mm) inserted into the eye just below the cornea A second electrode (Ag AgCl) served as reference electrode which was placed in contact with the filter paper which was connected to ground In spectral sensitivity measurements the relative light intensity (in photons) necesscary to elicit a potential change of constant amplitude (50 μV) was determined at wavelengths between 350 and 650 nm Intracellular recordings were made with glass microcapillaries with impedances ranging between 30 to 50 megohms when filled with 3 M KCl The potentials were fed into a preamplifier (Grass P 6—12) and displayed on an oscilloscope (Tektronix 502)

Results

Fig 1 shows the response recorded from the photoreceptor layer of a moth reared on the retinol deficient diet. The optic lobes had been removed The shape of the response was similar to that found in moths reared on a normal diet An initial transient phase of the potential change was usually seen in response to stimulus intensities stronger than that used in the illustrated experiment

Fig 2 shows an electroretinogram recorded from a retinol deficient eye with intact optic lobes A positive and a negative potential change are seen at the beginning and end of stimulation respectively The potential changes are probably due to activity



Fig 3 Intracellularly recorded responses to light stimulation of eye from moth reared on retinol deficient diet Horizontal bar 200 ms Vertical bar 1 mV Positivity upwards

in the optic lobes (cf Bernhard 1942, Autrum and Gallwitz 1951) Cessation of the light stimulus was followed by a transient increase in the negative potential followed by a slow decay

Intracellular recordings showed that the membrane potentials of photoreceptor cells from retinol deficient moths ranged from 20 to 70 mV The membrane potentials were usually less stable than those recorded from the photoreceptors of normal eyes Intracellularly recorded receptor potentials from deficient moths are seen in Fig 3 The configuration of the receptor potentials was similar to that recorded from normal cells

Fig 4 illustrates the increase in amplitude of the extracellularly recorded potential as a function of stimulus intensity (white light) in both deficient and normal eyes The slopes of the curves are not significantly different ($p > 0.10$)

The spectral sensitivity of eyes in deficient moths and, for comparison, that of eyes in normal moths (Hoglund and Struwe 1970), are illustrated in Fig 5 The spectral sensitivity of deficient eyes correlates well with that of normal eyes

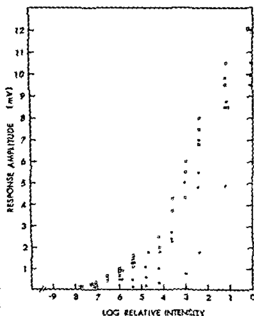


Fig 4 Relation between response amplitude and log relative stimulus intensity for 3 normal eyes (filled circles) and 5 eyes of moths reared on retinol deficient diet (open squares) Extracellular recording

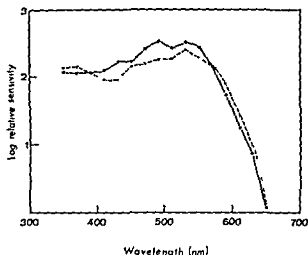


Fig 5 Spectral sensitivity of normal eye (broken line) and eye of moth reared on retinal deficient diet (continuous line) Extracellular recording (Values for normal eyes from Hoglund and Struwe 1970 Reproduced with permission from Springer Verlag Berlin—Heidelberg—New York)

Discussion

The present study provides evidence that the configuration and amplitude of extracellularly and intracellularly recorded potentials from eyes of moths reared on a diet deficient in retinol are similar to those of normal eyes The spectral sensitivity of deficient and normal eyes are also similar Electronmicroscopical studies of retinol deficient moths (Carlson Gemne and Robbins 1969) show that the retinular cells are structurally deranged Similar observations have been made on the eyes of retinol deficient vertebrates (Dowling 1964) There are however, observations which indicate that some of the receptors in the moths reared on retinol deficient diet have normal structure (Carlson and Gemne, unpublished observation) The receptors which do not suffer structural derangement are relatively few They can therefore probably generate only part of the extracellular response recorded in the present study

Previous studies on flies (Goldsmith Barker and Cohen 1964, Goldsmith and Fernandez 1966) and mosquitoes (Brammer and White 1969) show that retinol deficiency is associated with a decrease in photosensitivity (flies) and in amplitude of the response to light stimulation (flies mosquitoes) The normal receptor response to light of the moth eyes in the present study suggests that the moth photoreceptors were less deficient than those of the flies and mosquitoes Aseptically reared retinol deficient flies are less sensitive to light than those reared septically (Goldsmith and Fernandez 1966) A less severe retinol deficiency in the moths in the present study, as compared to the flies and mosquitoes may therefore be related to the rearing conditions

In retinol deficient rats a decrease in rhodopsin content is correlated with an increase in the threshold of the electroretinogram, and a later decrease in the amount



Fig ... worm moth, *Man* ... m of plant-reare ... with well-defir ... rhabdomeric

of opsin causes structural changes in the rod outer segments (Dowling 1964) In the moth, structural changes of the photoreceptors seem to precede a decrease in threshold This difference between insects and vertebrates may be caused by different biochemical properties of the photopigments

The results of the present study indicate that the amplitude and configuration of the receptor potential, and the sensitivity to light of structurally deranged photoreceptors are similar to those of normal receptors

We thank Dr William E Robbins, USDA ARS, Beltsville, Maryland, USA for rearing the moth larvae and supplying the pupae This investigation was supported in part by a Special Fellowship F 11 NB 1867—01 VSN to S.D Carlson from the National Institute of Neurologic ... the Air Force Office of Scientific Resear ... European Office of Aerosp Research ... Medical Research Coun Stiftelsen

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Acta physiol. scand. 1972. 84. 295—305

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Changes in Precapillary Resistance in Skeletal Muscle Vessels Studied by Intravital Microscopy

By

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Abstract

Eriksson, E. and B. Lisander. *Changes in precapillary resistance in skeletal muscle vessels studied by intravital microscopy.* Acta physiol. scand. 1972. *84.* 295—305.

The microcirculation of the cat's tenuissimus muscle was observed with particular regard to internal arterial diameters, linear flow rates and capillary flow distribution. Simultaneous recording
carried
dilator d
a slight
diameter
were no
steady tone. Nevertheless, capillary flow patterns changed perpetually. Capillary contents were stagnant and flow even reversed sometimes due to transient trapping of passing leucocytes. Maximal drug induced dilations caused 50 % diameter increases in arteries of 15—100 μ, with smaller changes in larger ones. Intense vasoconstrictor fibre activation lowered flow 6—8 times and reduced diameters in arteries of 15—200 μ with a maximum at the 30 μ size, where closure could occur. Luminal reductions were absent in immediate precapillary sections. During stimulation a marked leucocytosis was observed in the microvessels. It is concluded that in the muscle studied, lacking morphologically distinct precapillary sphincters, variations in capillary blood flow and in the perfused capillary surface area are brought about by luminal changes of end arterioles, to which white cell obstruction of microvessels may contribute. Further, the relative importance of the resistance of the different series-coupled sections may vary considerably with the regional tone. Sections responsible for changes in resistance and in capillary flow distribution are evidently not distinctly separated morphologically.

The vascular bed of skeletal muscle, supplying 40—50 % of the cat's lean body mass, has been thoroughly studied with various techniques. Estimations have been made of blood flow resistance, regional blood volume, capillary flow distribution, and transcapillary exchange in terms of diffusion and filtration. Although it has become increasingly clear that all these aspects of vascular function may be profoundly influenced by vascular smooth muscle activity, the exact sites of the underlying luminal adjustments are still largely unknown. A combination of morphological and physiological approaches is a prerequisite for further clarification of these problems

Thus, in the present study, recording of the regional flow resistance has been combined with intravital observation of the microcirculation in a thin sheet of striated muscle, the tensor of the crural fascia (the tenuissimus muscle) The observation technique as well as the detailed anatomy of this vascular bed will be described elsewhere (Brånemark and Eriksson Eriksson and Myrhage, to be published) Stimulation of the lumbar sympathetic chain and intraarterial administration of vasodilator drugs have been utilized to induce changes in vascular smooth muscle tone Attention has been focused upon changes in the precapillary parts of the vascular bed A preliminary report of the present observations has been published (Eriksson and Lisander 1969)

Methods

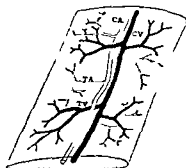
In thirty five cats anesthetized with chloralose following induction with ether, the tenuissimus muscle was exposed for observation in transmitted light in accordance with the method described by Brånemark and Eriksson (to be published) The tenuissimus muscle, characterized as a fast muscle (Lewis and Proske, personal communication) was observed under the microscope in ordinary mixed white light from which the infra red component had been filtered off A green filter with maximum transmission at 5500 Å was used to minimize damage of the observed tissue About 1 cm^2 of the muscle was observed during the experiment Objectives (X4 NA 0 14 X10 NA 0 30, X23 NA 0 55, X55 NA 0 84, X75 NA 1 00) giving magnifications from 40 to 750 times were used Internal vessel diameters could be measured with an optical micrometer Linear flow rates were calculated with a flying spot method (Brånemark and Jonsson 1963) Different vessel segments were photographed and microcirculatory sequences of interest were recorded on videotape

The jejunum ileum and colon were extirpated and the homolateral sympathetic chain was isolated and cut centrally The chain could be directly stimulated at supramaximal intensity and different frequencies (1—20 imp/s) This was usually done after atropine was administered at 1 mg/kg b w i v as to block cholinergic vasodilator fibres In some cases the sympathetic chain was initially left intact and when effects of autonomic denervation were to be studied a thread around the chain was pulled during simultaneous observation of the vessels The inferior mesenteric artery was cannulated and acetylcholine and papaverine were administered to the hind limbs by this route The venous outflow from all the muscles in the hindleg was continuously measured with an optical drop recorder in the femoral vein The paw was excluded by tight ligation Heparinization was necessary for this recording (Heparin Vitrum 10 mg/kg b w i v) A cannula was inserted in the subclavian artery and via a transducer (Statham P23AC) blood pressure and heart rate were recorded on a Grass polygraph together with muscle blood flow

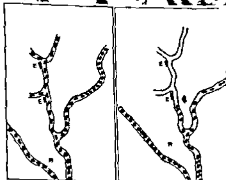
Results

The microcirculation of the transilluminated tenuissimus muscle was observed in combination with recording of systemic arterial blood pressure and blood flow in the hindlimb in most experiments The high degree of optical resolution obtained can best be judged by Fig 2 and 3 The muscle preparations remained in good condition in spite of the heparinization usually necessary neither showing a tendency to edema formation nor exhibiting large numbers of leucocytes sticking to the vascular walls The microcirculation remained essentially unchanged even after 8 hours of observation

Fig 1 shows the general arrangement of the microvessels in the tenuissimus muscle From the central arterial and venous vessels (50—100 μ in internal diameter) the tranverse arterioles and venules emerge forming endarterioles" and 'end-



F g 1 Schematic representat on of vascular arrangement in the tenuiss mus muscle CA = central artery CV central vein TA = trans erse arteriole TV = transverse venule



F g 2 Pre ap llary sec on of skeletal muscle vessels a) dur ng rest and b during sym pathetic stimulat on Ne ther precap llary sphincter acti ity no luminal reduct ons were observed during sympathetic stimulation In b) some vessels (above arrow) do not conta n any blood cells due to an upstream contrac t on of arterioles R red blood cells E = nucleus of an endothel al cell

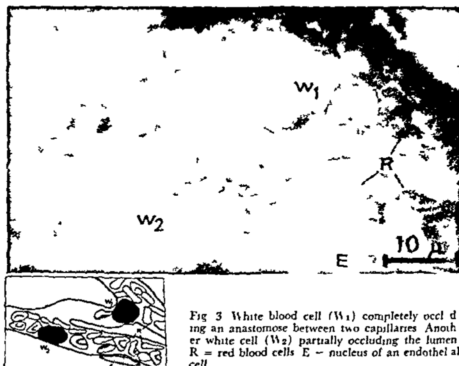


Fig 3 White blood cell (W1) completely occl d ing an anastomose between two capillaries Anoth er white cell (W2) partially occluding the lumen R = red blood cells E – nucleus of an endothel al cell

venules (20—50 μ in internal diameter) Occasional arteriovenous shunt like vessels with contractile walls about 15 μ in diameter and 500 μ in length could be ob served in skeletal muscle tissue in some preparations (in about 1 out of 20) No anastomoses between branches of similar size could be seen in the aborizations of the transverse vessels except that the capillaries were interconnected at about every 200 μ by channels 5 μ in diameter and 30 μ in length The arterial ends of the capillaries exhibited the narrowest lumina in the vascular bed Further vascular dimensions are given in Fig 4 No luminal reductions indicating the existence of 'sphincters were observed in the immediate precapillary sections either under rest ing conditions or during sympathetic stimulation Vessels exhibited a fairly steady tone *Significant rhythmic contractions were not seen under resting conditions*, although in smaller vessels diameter changes of less than 5 per cent might have passed unnoticed In spite of this there were dramatic variations of flow in individual capillaries and linear flow rates could range from standstill to 1—2 mm/s It ap peared as if the fluctuations in capillary flow were sometimes brought about by white cells obstructing the loops Such leucocyte plugging could impair flow for varying lengths of time from a few seconds up to several minutes see Fig 3)

In 10 expts the microvessels were observed during a sudden mechanical inter ruption of the ipsilateral sympathetic chain After about 5 s this initiated a vaso constriction which was usually of the same degree as that evoked by maximal sympa

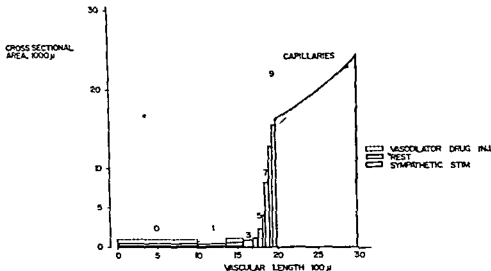


Fig 4 Denervated vascular bed Horizontal axis Length of one transverse arteriole and the mean lengths of its successive arborizations Vertical axis Total cross sectional area for each vascular generation Thus, the area of each block representing one vascular generation is proportional to its blood volume The numbers of the generations are indicated above the blocks starting with the transverse arteriole denoted 0 Mean diameters for the vascular generations are 27 μ, 18 μ, 15 μ 12 μ, 10 μ 10 μ, 9 μ, 9 μ, 8 μ, 6μ and, for the capillaries, 4 5 μ at their arterial ends and 5 5 μ at their venous ends Note that only the transverse arteriole and its two first generations of branches are affected by acetylcholine and sympathetic stimulation

thetic stimulation after about 15 s (see below) The vessel diameters then returned to, but not beyond, resting values After this procedure, flow in the hind limb had risen from around 5 ml/min and 100 g tissue to 7—10 ml/×min 100 g suggesting a slight relaxation of larger vessels

I a injections of 1—4 μg acetylcholine into the denervated vascular bed caused biphasic responses from the microvessels, although the blood flow of the limb at the same time rapidly increased to 35—40 ml/min×100 g at most During the first 30 s after injection, the microvessels displayed a slight constriction followed by a pronounced dilation The latter could amount to a 50 % luminal widening in arteriolar vessels of 15—50 μ

Papaverine was given in doses of 5 or 10 mg by the same route Both doses gave the same effect in all the observed vessels Arterioles of 15—50 μ increased their diameters by 50 % and larger arterioles by about 10 % Maximal effects were seen 10—15 s after injection and remained for at least 15—20 s no biphasic responses were seen Blood flow through the limb reached values indicating that maximal dilation of the vascular bed had occurred (35—40 ml/100 g × min)

Arterial vessels with internal diameters between 10 and 200 μ were observed during sympathetic stimulation at frequencies up to 20 imp/s Reduction of ve diameters was seen within 5—15 s of stimulation and maximal effects were

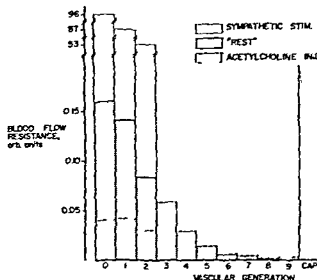


Fig. 5. Same vascular bed as in fig. 4. Flow resistance is calculated for each vascular generation in arbitrary units under assumption that all vessels are open for flow. For further explanations see text.

after 30 s. Vasodilation upon stimulation was never seen, regardless of whether atropine had been given or not. During prolonged stimulation the vasoconstriction remained for 120 s, after which the vessels relaxed for about 30 s. The constriction again became maximal after this "breakthrough". The breakthrough phenomenon was then often repeated in a rhythmical pattern at intervals of 30 s. With stimulation of 8—20 imp/s internal luminal reductions ranged between 40 and 90 % in vessels smaller than 50 μ. Total interruption of flow was common (Fig. 2). No diameter changes could be seen in vessels of about 10 μ or less. In vessels over 50 μ the percentile constriction decreased gradually with increasing internal diameter being about 25 % in arteries of 200 μ, vessels feeding the central artery. Linear flow rates increased during the first few seconds of stimulation, but when vascular constriction became evident they decreased rapidly. Upon stimulation with 8 imp/s or more flow could stop completely in some vessels. In others only plasma flow was seen as indicated by the passage of thrombocytes. During intense vasoconstriction leukocytosis of various degrees was observed in the microvessels with a clear maximum 8—15 s after the start of stimulation. However, the cells were swept away during the breakthrough periods or when stimulation was interrupted. Plasma skimming occurred and hematocrit in the vessels observed always decreased during stimulation (see Fig. 2).

Fig. 4 shows cross sectional area, length and volume of one representative transverse arteriole and its arborizations. The branching was dichotomous throughout this part of the vascular bed, as was the case with most transverse arterioles. Total cross sectional area increased by a factor of 1.1—2 at each arteriolar branching except for the most proximal one. The intravascular blood volume in these microvascular compartments was largest in the capillaries, which have slightly larger

diameters at their venous ends The total volume of the precapillary microvessels that responded actively to sympathetic stimulation was only some 4 per cent of that contained in the total compartment studied from the central artery to the distal end of the capillaries For this reason, sympathetic stimulation could expel only 2—3 per cent of the blood volume in the entire microvasculature under study, the blood volume increase by acetylcholine was of the same magnitude

Fig 5 represents a calculation of the flow resistance of the same consecutive segments according to the law of Poiseuille No attention has been paid to variations in blood viscosity, which appears to range from about 2 5 cP in the larger vessels observed to about 1 5 cP in the capillaries (see Bayliss 1952 and Folkow and Neil 1971) However, such differences would only moderately affect the calculations, they would not alter the general tendencies found The resistance of consecutive vascular generations decreased towards the capillary level Only the first three vascular generations appeared to be directly affected by acetylcholine or sympathetic stimulation The resistance of the transverse arteriole appeared to decrease by 75 per cent after acetylcholine or to increase several hundred times under the impact of intense vasoconstrictor fibre activity

The central artery, 85 μ in diameter, was fed by two vessels about 40 mm apart in the particular experiment shown in Fig 4 and 5 Hence, the blood had to travel at least 10 mm before it reached the "mean' transverse arteriole This distance would impose a resistance of about one tenth that of an individual transverse arteriole Since there were as many as 40 transverse arterioles in these 40 mm, the resistance of the central artery was most important It exceeded that of all the parallel coupled transverse arterioles taken together by a factor of four However, sympathetic stimulation exerted its most marked effects upon vessels smaller than central artery This caused the resistance of all the transverse arterioles combined to exceed that of the central artery by about 2—3 times

If the resistance of the precapillary branches of the transverse arterioles was added to that of these vessels at rest the sum was about equal to the figure for the central artery During sympathetic stimulation however, it could exceed that of the central artery by a factor of 4—10 Clearly, the neurogenic vasoconstriction tended to increase the relative importance of the resistance of smaller precapillary vessels in the vascular compartment studied, at least in the initial phases of a neurogenic resistance increase

Discussion

The arrangement of the microvessels in the tenuissimus muscle will be described in detail in another publication (Eriksson and Myrhage to be published) A few points, however, deserve comment here As shown in Fig 1 the central arterial and venous vessels run parallel to the muscle fibres and close together From these vessels arterioles as well as venules branch off at right angles to the muscle fibres and from each such transverse arte[illegible]

feeding the capillaries, which are again parallell to the muscle fibres No morphologically clearcut thoroughfare channels could be observed as described by Zweifach and Metz (1955) for the spino trapezius muscle Arterio-venous shunt like vessels were only seldom encountered in skeletal muscle tissue proper, in keeping with earlier morphological (see Illig 1961) and functional studies (Piper and Rosell 1961 Folkow, Mellander and Öberg 1961)

Cutting the sympathetic chain with due time allowed for stabilization, caused no visible diameter changes in the microvessels observed After this procedure the blood flow of the hind limb was around 7—10 ml/min$\times$100 g, a value only slightly exceeding that for the muscle vessels when normally innervated One feature indicating a considerable myogenic (Folkow 1964) tone after autonomic denervation was that i a injections of acetylcholine or papaverine could increase muscle blood flow up to 35—40 ml/min and 100 g tissue This was accompanied by a marked dilation of vessels in the tenuissimus muscle, especially in arterioles of 15—50 μ diameter Larger vessels dilated comparatively little in keeping with the notion that their inherent tone is less and with earlier observations that tone of smaller vessels in the arterial tree is least affected by sympathectomy (see Wideman 1963) Stimulation of the sympathetic chain produced a marked vasoconstriction This was most pronounced in the transverse arterioles and their first generations of branches At 8 imp/s or slightly below, maximal constrictions could be obtained That vessels of similar dimensions are reached by adrenergic nerves has been shown by Fuxe and Sedvall (1965) and Bolme and Fuxe (1970)

In accordance with commonly accepted terminology (Mellander and Johansson 1968) the skeletal muscle vascular bed studied here may be functionally subdivided into pre- and postcapillary resistance vessels, capacitance vessels and precapillary sphincters if the exchange vessels are exluded from consideration In the following an attempt will be made to determine what the morphological counterparts are of these functionally defined vascular sections

It has been suggested that arterial vessels larger than 40 μ in internal diameter are responsible for approximately half of the vascular resistance within skeletal muscle tissue (Djojosugito *et al* 1970) Further, the mean capillary pressure in isovolumetric equilibrium may be estimated at perhaps 15—25 mm Hg (see Landis and Pappenheimer 1963) Such figures suggest that between 25—50 % of the total flow *resistance in skeletal muscle tissue may be exerted by the microvascular* compartment observed here ranging from the central arterial vessel with an internal diameter of 50—100 μ to the venular ends of the true capillaries An attempt was made to deduce the distribution of flow resistance along these vessels by means of the law of Poiseuille and actual dimensional measurements In the resting denervated state the two sections represented by the central artery and the transverse arterioles with their precapillary arborizations appeared to contribute about equally to the regional resistance However, as the transverse arterioles and their most proximal generations of branches could often be brought to closure during sympathetic stimulation, their relative contribution to the overall resistance of the microvascular compartment

increased greatly As will be discussed later, these particular vascular sections may even function as precapillary sphincters by virtue of their marked luminal reductions

There is abundant evidence that in skeletal muscle tissue, the capillary filtration coefficient (CFC), a measure of hydrodynamic conductivity, and its diffusion counterpart, the PS value, can be changed markedly by influences known to affect smooth muscle contractility Vascular smooth muscle elements are therefore generally held to be responsible for such changes at least in situations where alterations of capillary permeability can be excluded (see Mellander and Johansson 1968) The term "precapillary sphincter' was used in 1944 by Chambers and Zweifach to designate the muscular investment at the origin of the branches from the preferential channel According to Zweifach and Metz (1955) such structures are likely to be present in skeletal muscle as well On the other hand Algire and Merwin (1955) ascribed changes in the capillary blood flow in this vascular bed to variations in arteriolar tone In the present preparation morphologically clearcut precapillary sphincters were not observed The smallest precapillary vessels were apparently devoid of contractile elements, for they never exhibited luminal changes regardless of whether vasoconstrictor stimulations were performed or vasodilator drugs given

However, as mentioned, vasoconstrictor fibre activation was found to reduce the diameters of some precapillary vessels greatly The maximal effect usually occurred in arterioles of about 30 μ in diameter In these, total occlusion of the lumina was often seen with intense sympathetic stimulation These strong constrictions could induce a complete standstill and must have implied a decrease in both the diffusion and filtration capacity of the vascular bed Therefore these arteriolar sections seem to be able to function effectively like proximally placed "precapillary sphincter regions' each influencing a group of capillaries rather than individual ones Thus diffusion distances may be dramatically increased, creating situations of profound functional shunting , even more so than in a one capillary—one sphincter arrangement.

It also appeared as if the contents of the capillaries were brought to a standstill sometimes by white cells obstructing the loops Flow began again after a few seconds up to several minutes White cells are less pliable than erythrocytes and it seems possible that they could temporarily obstruct the lumina when the pressure gradient is low Pressures between 2 and 40 mm of water may be required to make a single white cell pass a glass capillary with a lumen of 5 μ (Adell Skalak and Brånemark 1970) Thus, leucocytes must be regarded as elements which may critically affect flow through the capillaries and adjacent microvessels This is especially so since such cells tend to pool in capillary beds (see Bierman Kelly and Cordes 1965) and since the entrances of the capillaries possess the smallest lumina in the vascular bed studied

During sympathetic stimulation hematocrit in the microvessels decreased and at the same time white cells appeared to accumulate These latter cells were again swept away with the blood stream simultaneously with the vascular relaxation after

stimulation In view of these observations, it is tempting to assume that true closure of the microvessels elicited by smooth muscle contraction may be greatly facilitated by the presence of blood corpuscles, notably the leucocytes

The sympathetically induced leucocytosis in the microvessels, which was too pronounced to be explained by a mere "skimming" of cells in connection with the vasoconstriction, occurred with a very short latency Since the nerv.. st nulations only affected a restricted part of the animal, the most likely explanation to this phenomenon appears to be a release of white cellls previously sticking to the upstream vascular walls (see Athens *et al* 1961) Such a leucocyte mobilization may be of importance for the initial circulatory events during sympathetic activation.

To summarize, a more or less complete closure of 'end arterioles" in the vascular bed must serve the same purpose as "true' precapillary sphincters The present ex periments suggest that these end arteriolar sections functionally serve as "sphincters" in the vascular bed of skeletal muscle In addition to such a mechanism, it is sug gested that leucocytes can impair flow for varying lengths of time through individual capillaries or groups of capillaries Thus, by means of a temporary luminal plugging they could potentiate the sphincter like function of the end arteriolar sections

With regard to the capacitance function of the microvascular compartment studied it is clear from Fig 4 that its small blood content is decreased by only 2—3 per cent with maximal sympathetic stimulation. Hence, the major part of blood mobilization from this tissue during sympathetic stimulation, estimated at maximally about 30 per cent of the regional blood volume (see Mellander and Johansson 1968) must emanate from other vascular sections, notably from the veins The reason for this low figure of regional blood mobilization from precapillary microvessels seems to be that those sections responding actively to sympathetic stimulation contain only a small fraction of the blood content of this compartment Therefore, intense vasocon striction at this point would increase flow resistance considerably but would only produce a minor expulsion of blood

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The Oxygen Supply to the Retina, II. Effects of High Intraocular Pressure and of Increased Arterial Carbon Dioxide Tension on Uveal and Retinal Blood Flow in Cats

A study with radioactively labelled microspheres including flow determinations in brain and some other tissues

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Abstract

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15 µ and 35 µ microspheres labelled with ^{85}Sr and ^{169}Yb were used to determine the rate of blood flow through various intraocular tissues, optic nerve, brain, kidney cortex and small intestine in cats at normal and increased P_{aCO_2}. One eye had its spontaneous intraocular pressure, the other eye had its pressure stabilized at a higher level. At normal P_{aCO_2} a reduction in perfusion pressure resulted in decreased vascular resistance in the iris, the ciliary body and the retina, but not in the choroid. In the retina the eye with reduced perfusion pressure had a significantly higher blood flow than the control eye. Increased P_{aCO_2} resulted in increased blood flow in all ocular tissues and all extraocular tissues studied except the kidney cortex. Reductions in perfusion pressure at high P_{aCO_2} resulted in further decreases in vascular resistance in two eyes of seven only.

The results suggest that about 21 % of the O_2 consumed by the retina is delivered by the retinal blood vessels, the rest by the choroid. Both *myogenic* and *metabolic mechanisms* seem to contribute to the adjustment of retinal vascular resistance after a change in perfusion pressure.

In a previous study (Alm and Bill 1972), we described a procedure which under certain conditions can be used to obtain continuous semiquantitative information about changes in blood flow through the retina. The method — to measure the P_{O_2} in the vitreous body close to the retina, P_{VIO_2}, — indicated that in cats retinal blood flow is autoregulated within a wide range of perfusion pressures, defined as the mean arterial blood pressure (MAP) minus the intraocular pressure (IOP), and that increases in the arterial P_{CO_2} (P_{aCO_2}) result in marked increases in blood flow through the retina. In the experiments reported here we have tried to make quantitative determinations of the blood flow through the retina as well as that through the different parts of the uvea. The perfusion pressure was at a normal level in one eye and at a

low level, still within the autoregulatory range, in the other The perfusion pressure was reduced by stabilizing the IOP at a high level Some experiments were performed at a normal P_{aCO_2}, others at an increased P_{aCO_2}

To determine the blood flow quantitatively we have used radioactively labelled microspheres Such spheres of different composition and size have been used in many studies on the blood flow through various tissues (see Kaihara *et al* 1968, Neutze, Wyler and Rudolph 1968, Wagner *et al* 1969, Flohr and Hoppe 1969, Roth *et al* 1970 and Lunde and Michelsen 1970) The microspheres are injected into the left atrium or ventricle of the heart After mixing with blood in the heart and the aorta the particles follow the blood stream and are trapped in the various tissues in proportion to the blood flow One great advantage of this method is that it can be used to determine blood flow in inaccessible tissues and in very small parts of tissue Microspheres used for blood flow determination in many tissues usually have a diameter of 35—50 μ Such spheres are stopped in arterioles of this size Smaller particles with a diameter of about 15 μ are stopped in smaller blood vessels and therefore give a much better spatial resolution if the same total mass of particles and nuclide are used Small particles, however, tend to pass through the vascular beds studied

After some pilot experiments with labelled 35 μ particles it was clear that to keep the total mass of particles injected at a reasonable level smaller particles had to be used This was necessary to avoid the statistical problems that are due to chance differences in particle concentration in very small volumes of plasma Other pilot experiments, where a mixture of labelled 35 μ particles and labelled albumin was injected into the carotid artery and where venous blood samples were taken from the uvea, indicated that more than 99 % of the particles that entered the uvea were retained there for more than one min For 15 μ particles the corresponding figure was about 95 % To justify the use of the smaller particles some of the experiments reported here were performed with a mixture of small and large particles

Methods

Adult cats of both sexes weighing between 1 8 and 4 1 kg were used Anesthesia was induced with

Instrument Inc Palo Alto Cal) (see Alm and Bill 1970)

Heparin 500 I U /kg b w was given i v to prevent clotting The animal was placed supine on a heating pad The body temperature was determined with a rectal thermometer and kept close to 37 5° C

The anterior chamber of both eyes was cannulated with steel cannulae connected to a pressure transducer (EMT 490 A) for intermittent measurements of the actual IOP One of the anterior

injection of the microspheres

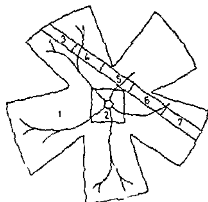


Fig 1 To study the regional blood flow/min/mm² in the retina and the choro d the flat

papilla removed, pieces number 3 and 7 represent the peripheral parts and pieces number 4—6 the area centralis and its surroundings

For injection of the microspheres a *thoracotomy was made and* a steel cannula connected by a polyethylene tubing to a syringe was introduced into the left ventricle of the heart The experiments were performed in two *series, in 13 animals the* P_{aCO_2} was kept at normal values and in 8 animals *it was increased to levels above* 60 mm Hg through ventilation with a mixture of room air and CO_2 In 5 of the normocapnic animals a mixture of 15 μ and 35 μ particles was used, in the remaining normocapnic and in the hypercapnic animals only 15 μ particles were used The suspension of the

the choroid sclera on the other After air-drying these preparations were used to make autoradiographs A *thin sheet of plastic* was placed between the preparations and the film (Agfa Gevaert Structurix D7-film) to prevent chemographic effects The exposure time for the retina preparations was 72 h

flo

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area centralis and its surroundings could be estimated.

Microspheres

pheres labelled with ^{85}Sr and 35 ± 5 μ

Minnesota) The spheres were used as

80 added to prevent aggregation The

Assay and calculations

The activity of the tissues and blood samples was determined by gammaspectrometry, when both types of spheres were used two channel gammaspectrometry was used to determine the activity due to each isotope in each sample Analysis of the blood samples collected from the femoral artery dur-

Statistics

In the following mean and S E are given Group comparison with Student's t test was used to determine the statistical significance of differences in blood flow between normocapnic and hypercapnic animals for the choroid the total uvea the small intestine and the kidney cortex For the other tissues an F test showed statistically significant differences in variance between the two groups ($p<0.05$) and therefore a nonparametric test, the Mann Whitney U test was used for these tissues (Siegel 1956)

Results

At the time of the injection of the microspheres the following data were obtained for the 13 animals ventilated with room air MAP 153±7 cm H_2O (n = 13), spontaneous IOP in the control eye 21 0±2 2 cm H_2O (n = 13), arterial P_{O_2}, P_{CO_2} and pH 95±2 mm Hg (n = 13), 26 1±0 9 mm Hg (n = 13), and 7 43±0 02 units (n = 12) respectively For the animals ventilated with a mixture of room air and CO_2 the values were, MAP 188±15 cm H_2O (n = 8), IOP 31 9±6 4 cm H_2O (n = 7), arterial P_{O_2}, P_{CO_2} and pH 85±4 mm Hg (n = 8), 80 9±4 3 mm Hg (n = 8), and 7 10±0 04 units (n = 6) respectively

5 expts were performed with injection of a mixture of 15 μ and 35 μ microspheres In the rest of the experiments only 15 μ microspheres were used The ratio (flow calculated from the data for the small spheres)/(flow calculated from the large spheres) was 1 02±0 04 in the choroid, 0 94±0 11 in the iris, 0 96±0 05 in the ciliary body, 0 98±0 04 in the kidney cortex, 1 03±0 08 in the brain and 1 07±0 15 in the small intestine The blood flow through the retina was too small to permit comparisons worth while only a few large microspheres were found there and the ratios varied from 0 5 to infinity

The blood flow values given in the following tables and figures were all calculated from the 15 μ microsphere data

Table I shows the blood flow through the different tissues of the eye with spontaneous IOP and some extraocular tissues in 13 normocapnic animals During the preparation of the ciliary body and the retina it proved very difficult to remove all rests of the vitreous body Water constitutes 99 % of the vitreous body Sullman 1951 To estimate wet weights of the tissues the retina and the ciliary body samples were therefore dried and reweighed The wet weights were calculated as dry weight 0 20 Dry weight/wet weight ratios of 0 20 were observed in brain tissue which suggests that the figure is appropriate for the retina In the iris the corresponding ratio was 0 21 which suggests that 0 20 is a reasonable approximation also for the ciliary body

Table II gives the same flow values for 8 animals ventilated with a mixture of room

TABLE I Normocapnic animals

Tissue	Weight of whole tissue (mg)	Blood flow in whole tissue (mg/min)	Blood flow (g/min per 100 g tissue)
Iris	65.7 ± 4.5 (n = 12)	60 ± 11 (n = 12)	97 ± 21 (n = 12)
Ciliary body	119.1 ± 4.5 (n = 10)	262 ± 30 (n = 12)	223 ± 27 (n = 10)
Choroid	57.5 ± 1.8 (n = 10)	734 ± 94 (n = 13)	1382 ± 185 (n = 10)
Whole uvea	242.3 ± 9.2 (n = 9)	1070 ± 122 (n = 12)	471 ± 74 (n = 9)
Retina	79.6 ± 4.6 (n = 10)	15 ± 2 (n = 13)	19 ± 3 (n = 10)
Optic nerve			14 ± 2 (n = 12)
Brain			37 ± 3 (n = 13)
Corpus callosum			18 ± 2 (n = 10)
Kidney cortex			334 ± 38 (n = 13)
Small intestine			37 ± 3 (n = 13)

TABLE II Hypercapnic animals

Tissue	Weight of whole tissue (mg)	Blood flow in whole tissue (mg/min)	Blood flow (g/min per 100 g tissue)
Iris	73.6 ± 4.3 (n = 7)	185 ± 34 (n = 7)•	245 ± 37 (n = 7)°
Ciliary body	138.8 ± 10.1 (n = 7)	750 ± 126 (n = 7)•	537 ± 77 (n = 7)°
Choroid	70.3 ± 3.6 (n = 4)	2165 ± 172 (n = 7)°	3504 ± 371 (n = 4)°
Whole uvea	284.0 ± 14.8 (n = 4)	3100 ± 274 (n = 7)°	1226 ± 138 (n = 4)°
Retina	83.7 ± 8.3 (n = 4)	56 ± 8 (n = 7)•	66 ± 17 (n = 4)•
Optic nerve			93 ± 14 (n = 6)•
Brain			339 ± 36 (n = 8)•
Corpus callosum			123 ± 13 (n = 8)•
Kidney cortex			429 ± 50 (n = 8)
Small intestine			59 ± 7 (n = 8)°

° Statistically significant increase in blood flow compared to normocapnic animals $p<0.01$ Student's t test

• Statistically significant increase in blood flow compared to normocapnic animals $p<0.01$ Mann Whitney U test

air and CO_2 for at least 15 min In this series the cannulation of the cornea failed in one of the animals Only flow values for extraocular tissues are included for this animal

The autoradiograph preparations revealed no regional differences in distribution of particles in the choroid or the retina The mean blood flow values in normocapnic animals at spontaneous IOP for different regions in the retina ranged from 0.043 to 0.047 mg/min/mm² and in the choroid from 1.14 to 1.50 mg/min/mm² The optic nerve head preparations were too small to permit reliable determinations of blood flow

After autoradiographs had been taken it was not possible to separate the sclera from the choroid Therefore in these cases the blood flow calculated for the choroid included that in the sclera The error introduced is minimal since other experiments showed that the blood flow through the posterior sclera is less than 2 % of that through the choroid

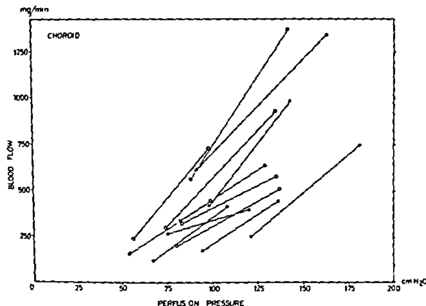


Fig 2—5 Radioactively labelled microspheres were used to determine the rate of blood flow in the choroid the ciliary body and the iris. One eye had its spontaneous intraocular pressure, the other eye had its pressure stabilized at a higher level The rate of flow in the different tissues and also in the whole uvea was plotted against the perfusion pressure This pressure was defined as the mean pressure in a femoral artery minus the intraocular pressure and expressed in cm H_2O Lines connect the points representing data for the two eyes of the same animal

O—O data obtained in experiments with both 15 μ and 35 μ spheres
●—● data obtained in experiments with only 15 μ spheres

Fig 2—4 shows the data obtained for flow in the different parts of the uvea in the normocapnic animals Fig 5 shows the results for the whole uvea in the same experiments The values for the two eyes were plotted against the perfusion pressure The values for eyes belonging to the same animal have been connected The perfusion pressure for the eye was defined as the MAP in the femoral artery minus the IOP The true perfusion pressure is somewhat lower since the pressure in the arteries entering the eye is lower than the pressure in the femoral artery In rabbits the difference is about 25 cm H_2O (Bill 1967) It is reduced by a rise in IOP

In tissues without autoregulation a simplified flow pressure diagram is represented by a straight line intercepting origin In the present experiments the true origin is somewhere around zero flow / 25 cm H_2O perfusion pressure (see above) Thus if in the experiments reported here the line connecting the values for the two eyes in the same animal is extrapolated and then intercept the positive pressure axis at values above 25 cm H_2O it suggests that there has been very little or no fall in vascular resistance in the eye with reduced perfusion pressure An interception of the positive pressure axis at values between 0 and 25 cm H_2O or of the positive flow axis suggests a fall in vascular resistance in the eye with reduced perfusion pressure In experi

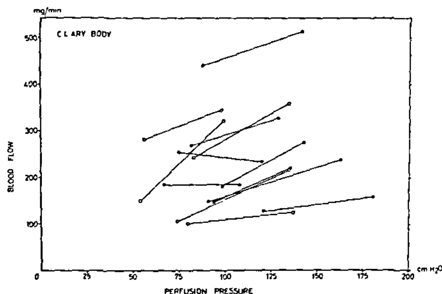


Fig 3

ments where the line connecting the two flow values for the same animal is parallel with the pressure axis the effect of the reduction in perfusion pressure on blood flow has been completely compensated for by the fall in vascular resistance

The blood flow through the choroid (Fig 2) was markedly reduced in eyes with artificially elevated IOP Extrapolation of the line connecting the values for the two eyes in all cases but two intercepted the pressure axis at values above 25 cm H_2O Thus there is no indication that a lowering of the perfusion pressure results in a reduction in the vascular resistance in the choroid

The blood flow through the ciliary body in eyes with elevated IOP was less than that in the control eyes in 12 cases of 13 (Fig 3) The average decrease in blood flow was 63.6 ± 14.8 mg/min ($n = 12$) for a reduction in perfusion pressure of 50.9 ± 2.8 cm H_2O ($n = 12$) (One eye excluded from statistics see below) The difference based on paired data was significant ($0.001 < p < 0.005$) In 9 cases extrapolation of the line connecting the dots representing data for the two eyes intercepts the flow axis This means that the reduction in perfusion pressure had resulted in a decrease in the vascular resistance

In the iris the blood flow was essentially uninfluenced by changes in the perfusion pressure within the range studied (Fig 4) A rise in IOP thus decreased vascular resistance A marked difference in flow was observed only in one case In this cat a very high rate of blood flow was observed in the control eye This may have been the result of an unnoticed trauma to the iris during the cannulation of the cornea Mechanical irritation of the iris is known to release vasodilatating substances (Ambache Kavanagh and Withing 1965) For this reason the anterior uvea of this eye has not been included in the statistics Fig 5 shows that total uveal blood flow calculated from the flow values for the different parts decreased for a rise in IOP without clear

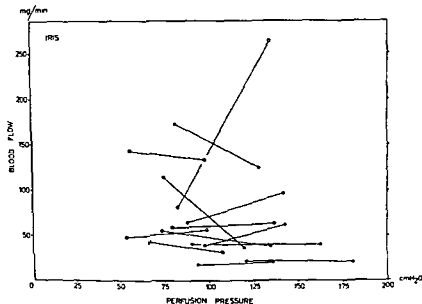


Fig 4

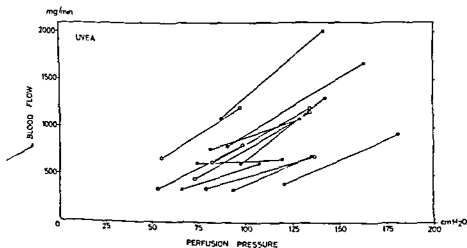


Fig 5

indications of decreased vascular resistance Thus reductions in the blood flow through the choroid conceal autoregulation in the iris and the ciliary body when the total uveal blood flow is determined

In the retina eyes with increased IOP showed a higher blood flow value in 12 cases of 13 (Fig 6) The average excess flow was 4 12±1 29 mg min (n = 13) for a reduction in perfusion pressure of 51 0±2 6 cm H_2O (n = 13) The difference based on paired data was significant (0 005<p<0 010)

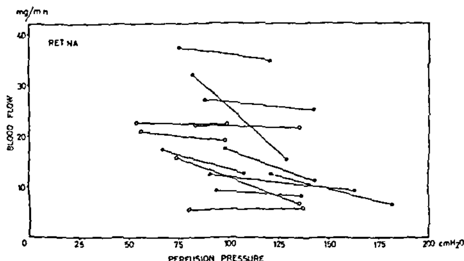


Fig 6 Blood flow through the retinas in cats ventilated on room air For explanation see Fig 2—5

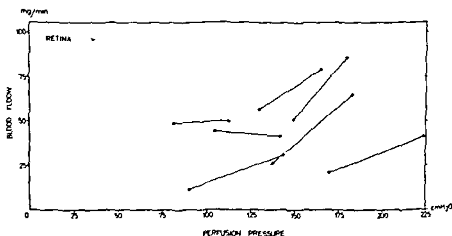


Fig 7 Blood flow through the retinas in cats ventilated on a mixture of room air and CO_2 For explanation see Fig 2—5

Fig 2—6 indicate that the flow values observed in experiments with injections of a mixture of large and small particles were not very different from those in experiments with only small particles A statistical analysis failed to show a statistically significant difference

High levels of P_{aCO_2} resulted in increased blood flow and limited ability to autoregulate the blood flow Fig 7 shows the result for the retina Here a reduction of the perfusion pressure resulted in a clearly decreased vascu[illegible] only in 2 cases out of 7 Similar results were obtained for [illegible] iris [illegible]

Discussion

The labelled microsphere method

In the experiments with injection of 35 μ and 15 μ microspheres the flow values calculated were not significantly different from those in which only small spheres were used This indicates that the dose of large spheres injected was not large enough to disturb the circulation appreciably Then it seems unlikely that the smaller spheres which tend to occlude smaller vessels could have much influence on the circulation in the tissues investigated, the total masses of small and large spheres injected were about the same It is known from previous studies (see Wagner *et al* 1969) that small particles tend to pass through the tissues The experiments with a mixture of 15 μ and 35 μ spheres indicate that with the procedure used in the present study such passage was very limited The blood flow of the retina was too small to permit a comparison between results obtained with large and small spheres In the brain, however, such a comparison showed that the small spheres were not lost to any great extent The retinal blood vessels are very similar to those of the brain, the diameter of the capillaries is less than 8 μ (Cunha Vaz, Shakib and Ashton 1966) This justifies the assumption that also in the retina the small spheres give a good estimate of the blood flow

Blood flow through different tissues at normal P_{aCO_2}

Previous experiments with direct determination of the blood flow through the whole uvea have shown that the flow is about 1 10—1 20 ml/min (Bill 1962a, Alm and Bill 1970) Thus the value obtained in the present study, 1 07±0 12 g/min, is in good agreement with the previous values The rates of blood flow through the different parts of the uvea have not been known previously the present experiments show that about 70 % of the uveal blood flow goes through the choroid, 5 % through the iris, and 25 % through the ciliary body The even distribution of blood flow within the choroid and the retina differs from that seen in monkeys In the cynomolgus monkey the blood flow through the central parts is 5—10 times larger than that through the periphery in both the choroid and the retina (Alm and Bill unpublished observations)

The blood flow through the choroid 1384 g/100 g/min, is very large It is about four times as large as that through such a well perfused tissue as the kidney cortex The flow through the ciliary body and the iris is less than that in the kidney cortex but much larger than that in the small intestine The flow value calculated for the retina underestimates the flow in the part of the retina in which the retinal blood vessels are distributed On the assumption that the retinal blood normally nourishes the inner two thirds of the retina the flow in this region is 29 g 100 g min a figure which is similar to the values obtained for mixed white and grey matter of the brain The blood flow through the optic nerve is similar to that in cerebral white matter represented by corpus callosum

The values presented here for brain tissue and kidney cortex are similar to those reported in studies with 50 μ particles (Roth *et al* 1970 Neutze Wyler and Rudolph

1968) The values for the small intestine is somewhat lower than the "resting value reported by Folkow, Lundgren and Wallentin (1963) for isolated, denervated small intestine of the cat, 40—60 ml/100 g tissue/min Differences in symphathetic tone and experimental conditions may explain the difference

Blood flow through different tissues at increased P_{aCO_2}

CO_2 is known to cause vasodilatation in brain (Reivich 1964), intestine (McGinn, Mendel and Perry 1967) and many other vascular beds including the uvea (Bill 1962b) In the present study the blood flow was increased in all tissues studied except the kidney cortex The unchanged flow in this tissue is probably the result of a central regulation of the distribution of the cardiac output since locally increased P_{CO_2} results in decreased vascular resistance in the perfused kidney (Lockett 1967)

In the uvea the different parts show a similar increase in blood flow, about 3 times that in the control group In a previous study (Alm and Bill 1972) it was shown that increased P_{aCO_2} increased the tissue P_{O_2} in the retina, which indicates a rise in blood flow A marked rise was found in the present study, the flow values in animals with increased P_{aCO_2} are 3 to 4 times higher than in the control group

The mechanism behind the vasodilatation caused by CO_2 is not known Evidences have been presented that the sensitive mechanism is situated in the vessel wall and not in the tissue (Severinghaus and Lassen 1967) and that it is mediated through changes in local pH rather than in P_{CO_2} (see Wahl *et al* 1970)

Effects of reduced perfusion pressure

Previous studies in cats have shown that immediately after a rise in IOP the blood flow through the uvea is reduced that there is a small recovery in flow and that the blood flow stabilizes below the initial level (Bill 1962c) The experiments reported here suggest that vasodilatation in the iris and the ciliary body account for the partial recovery of the flow In the choroid any change in vascular resistance appeared to be very small In the ciliary body the vasodilatation is not enough to give efficient autoregulation of the blood flow in the pressure range studied, but the flow in the iris seems to be very efficiently autoregulated

Studies on the P_{O_2} in the retina (Alm and Bill 1972) have suggested that the blood flow through the retina is autoregulated at perfusion pressures above 50—100 cm H_2O The experiments reported here confirm that a reduction in perfusion pressure results in a marked fall in vascular resistance in the retina In fact, the reduction is so pronounced that the blood flow through the retina in eyes with moderately reduced perfusion pressure is even higher than that in control eyes

In most tissues increased metabolic demands are met by vasodilatation probably caused by accumulation of metabolites in the tissue The factors involved in autoregulation of blood flow at varying perfusion pressures are less clear, metabolic mechanisms may account for part of the autoregulation Berne 1964) but there is also evidence in favor of a myogenic theory (Folkow 1964) In the brain the mechanism that maintains constant flow at varying perfusion pressures seems to be mainly myogenic

The arguments are that in the autoregulatory range there is not enough change in blood flow to produce significant alterations in P_{O_2} or local concentrations of metabolites (see Haggendal, Nilsson and Norback 1968), and that efficient autoregulation, although within a smaller range, is present even when blood flow has been increased through increased P_{aCO_2} or papaverin infusions (Ekström—Jodal, Haggendal and Nilsson 1970)

Autoregulation of the blood flow through a tissue may be lost due to surgical trauma or anoxia (Fog 1968), trauma to the blood (Folkow 1952) and reduced or abolished by anesthesia (Smith *et al* 1970) Since autoregulation was present in the experiments reported here in the iris and in the retina it is most unlikely that the lack of autoregulation in the choroid was due to loss of a normal response pattern The observation that a rise in P_{aCO_2} to a level above 60 mm Hg produced marked vasodilatation in the choroid shows that the responsiveness of the choroidal blood vessels to adequate stimuli had not been lost It is of some interest then to discuss the relative importance of metabolic and myogenic mechanisms for the autoregulation of blood flow through the different ocular tissues and to try to account for the lack of vasodilatation in the choroid at high IOP

In the retina the situation is complex due to the dual nutritional supply The present results and those of our previous study (Alm and Bill 1972) indicate that at a perfusion pressure reduced from the normal level to about 100 cm H_2O the retinal blood vessels are very much dilated at a P_{VrO_2} that is not significantly below the normal value Then the P_{CO_2} and the concentration of other metabolites must also be near the normal level. However, in the outer parts of the retina normally supplied by the choroidal blood vessels the P_{O_2} tends to decrease and the P_{CO_2} to increase The rise in P_{CO_2} in venous blood from the uvea is about 5 mm Hg when the normal perfusion pressure is halved (Alm and Bill 1970) Measurements of P_{VrO_2} at different P_{aCO_2} levels suggest that a rise of this order in the outer parts of the retina can be expected to have a moderate effect on the nearest retinal blood vessels Then one can speculate that after a rise in IOP there is a mainly myogenic autoregulation of the blood flow in the whole retina In the inner parts this makes the blood flow remain constant In the outer parts the increase in concentrations of CO_2 and other metabolites add a vasodilative stimulus that makes the flow increase above the normal level That myogenic factors play an important role is indicated by the observation that autoregulation was present in some experiments with high P_{aCO_2}

In the iris two factors tended to prevent a rise in the concentration of metabolites when the perfusion pressure was reduced These were the unchanged blood flow and an increased flow of mock aqueous humor through the anterior chamber This makes it seem very likely that in the iris autoregulation is mainly myogenic The autoregulation found in some experiments with high P_{aCO_2} supports this conclusion

It is not possible to draw any conclusions about the relative importance of the two mechanisms for autoregulation in the ciliary body Since the blood flow was reduced at least part of the vasodilatation observed may be accounted for by an increase in the concentration of CO_2 and other metabolites

It then remains to account for the failure of both metabolic and myogenic mechanisms to cause reduced vascular resistance in the choroid at increased IOP. The myogenic mechanism implies the existence of precapillary sphincters and/or rhytmic contraction and relaxation of the small resistance vessels, 'vasomotion' (see Folkow 1964). Precapillary sphincters also seem to play a major role in metabolic vasodilatation (see Mellander and Johansson 1968). Then it is interesting that direct observation of the microcirculation in the choroid has failed to provide evidence of either precapillary sphincters or 'vasomotion' (Friedman and Oak 1965).

The oxygen supply to the retina

The experiments reported here show that in cats the normal blood flow through the choroid is 734 mg/min or about 70 % of the total uveal blood flow. The normal a-v O_2 difference across the uveal tract in cats is about 1 vol %. Choroidal venous blood however is slightly more oxygenated than mixed blood from the choroid and the anterior uvea (Alm and Bill 1970), which suggests an a-v O_2 difference of about 0.8 vol % across the choroid. This means that about 5.6 μl O_2/min is extracted from the choroidal blood. The O_2 consumption in the choroid is not known, but a reasonable estimate (0.3 μl/min) can be used if we assume that the O_2 consumption is of the same order as that in rat aorta (1.03 μl/mg dry weight/h (Krantz, Jr, Carr and Knapp 1951). This leaves 5.3 μl/min for the O_2 consumption of the outer avascular part of the retina. The blood flow through the retina is about 15 mg/min. The O_2 saturation in the retinal veins is not known exactly but previous experiments (Alm and Bill 1972) suggest a value of about 40 %. Thus the O_2 extraction from the retinal blood vessels is about 1.4 μl/min in cats.

In vivo experiments in humans indicate that the O_2 consumption in the retina is about 10 ml/100 ml tissue/min (Andersson and Saltzman 1964). In the present experiments the mean dry weight of the retina was 16 mg corresponding to a mean wet weight of 80 mg (see above). With 5.3 μl/min from the choroidal blood vessels and 1.4 μl/min from the retinal blood vessels the O_2 consumption in the cat retina would be 8.4 ml/100 g tissue/min, a figure in good agreement with that calculated for humans.

A previous study has shown that increments in IOP have very small effects on the O_2 extraction from the blood perfusing the uvea as long as the blood flow is above 0.25 ml/min (Alm and Bill 1970). The present study indicates that a moderate rise in IOP may cause a small shift in the supply of oxygen to the retina, somewhat more coming from the retinal vessels.

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Storage of 5-Hydroxytryptamine in Rat Mast Cells. Evidence for an Ionic Binding to Carboxyl Groups in a Granule Heparin-Protein Complex

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Abstract

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[illegible]

5-HT release, as we previously showed for Hi release, is due to a cation exchange occurring on mast cell degranulation, when the amine-containing granules become exposed to cations of the extracellular milieu.

It was earlier proposed (Uvnäs 1964) that the release of histamine (Hi) and of 5-hydroxytryptamine (5-HT) from rat mast cells caused by degranulating agents might be the result of an ion exchange between the amines and extracellular cations in those mast cell granules which intra- or extracellularly became exposed to the extracellular fluid.

The matrix of the Hi-storing granules is mainly composed of an ionic complex of heparin and a basic protein (Lagunoff *et al.* 1964, Bergqvist, Samuelsson and Uvnäs 1971). In a recent paper (Uvnäs, Åborg and Bergendorff 1970) experimental evidence was presented to show that Hi is stored in ionic linkage to protein carboxyl groups in this complex (Fig. 1) and not, as previously assumed, to the granule heparin. This binding to weakly acidic groups explains why Hi is immediately and completely released from the granules as soon as they are exposed to cation-containing media, e.g. when mast cells degranulate or when isolated granules are suspended in isotonic salt solutions (Åborg, Novotny and Uvnäs 1967, Uvnäs *et al.* 1970, Röhlich, Anderson and Uvnäs 1971).

$$\text{Hep} \left\langle \begin{array}{l} COO^- \\ NHSO_3^- \\ OSO_3^- \end{array} \quad \begin{array}{l} H_3^+N \\ H_3^+N \\ H_3^+N \end{array} \right\rangle R - COO^- \quad H_3^+N - CH_2 - CH_2 - C \text{(imidazole: } C{=}CH{-}NH{-}CH{=}N\text{)}$$

Hep - HEPARIN

R = PROTEIN

Fig. 1 Assumed gross structure of the heparin protein histamine complex of mast cell granules at pH 7 (from Uvnäs *et al* 1970)

These and other observations from our laboratory (Nosal, Slorach and Uvnäs 1970, Fillion, Slorach and Uvnäs 1970) corroborate the above proposal as far as the release of Hi is concerned However, rat mast cells also contain 5-HT (Benditt *et al* 1955, Keller 1957), assumed to be stored like Hi in the basophil granules When the mast cells are exposed to Hi releasing agents both Hi and 5 HT are released (Garcia Arocha 1961, Bhattacharya and Lewis 1956, Moran, Uvnäs and Westerholm 1962)

It has been assumed that 5 HT, like Hi, is stored in ionic linkage to the granule heparin the argumentation likewise being the capacity of heparin to form a complex with 5 HT *in vitro* (Keller 1958) However, Sanyal and West (1959) found that 5 HT failed to form a complex with heparin *in vitro*

In the present study we will present experimental evidence to indicate that the releasable 5 HT in mast cells—like the Hi—is ionically linked to carboxyl groups (probably protein carboxyls) in the granule heparin protein complex 5 HT—like Hi —is shown to be released from its granule stores by cation exchange, when the granules are exposed to cation containing media

Methods

Quantitative determination of 5 HT and Hi

5-HT was determined according to Udenfriend *et al* (1955) Hi was assayed by the method of Shore *et al* (1959), except that the initial treatment with perchloric acid and the subse

upernatant used for the assays

Isolation of mast cells

Mast cells were isolated from the abdominal and thoracic cavities of male Sprague Dawley rats weighing 350—450 g as described by Thon and Uvnäs (1967) However after gradient centrifugation in F coll (30 and 40 %) the cells were not washed in isotonic sucrose solution unless otherwise indicated

Isolation of granules

Granules were obtained from water lysed mast cells After an initial centrifugation at 400 × g for 10 min to remove coarse material a second centrifugation was performed at 3000 × g for 20 min in order to precipitate the granules Usually granules were collected from 30 10^6—40 10^6 mast cells (15—20 rats) for each experiment For details of the isolation procedure see Uvnäs *et al* (1970)

Preparation of amine (and sodium) depleted granules

Granules isolated as described above were suspended in 10 mM sodium phosphate buffer, pH 7 (2 ml per rat) This results in an instantaneous exchange of the amines for sodium the amines occurring in the supernatant on subsequent precipitation of the granules by centrifugation at $3000 \times g$ for 20 min By washing these now sodium-charged granules 3 times in deionized water sodium was removed and 'depleted' granules obtained The yield of granules was about 0 2 mg (dry weight) per rat (For further details see Uvnas *et al* 1970)

Uptake of 5 HT, Hi and sodium

Depleted and dried granules were suspended in deionized water—about 0 15 mg granules per 100 µl—and aliquots of 100 µl pipetted into tared polypropylene tubes To these tubes were adde [illegible] belled amine or [illegible] s were centri fugec [illegible] ube with its preci [illegible] iating radio-activity The granule precipitate was then dissolved in 0 5 ml of 1 M NaOH and the radio-activity recorded in an aliquot by liquid scintillation spectrometry (For further details see Uvnas *et al* 1970)

5 HT

To 133 mg of 5 HT hydrogen oxalate was added 35 mg of $Ca(OH)_2$ in 25 ml of deionized water The precipitate was rapidly removed by centrifugation The supernatant was immediately adjusted to pH 7 with 0 2 N H_2SO_3 and diluted with deionized water to a 10 mM 5 HT stock solution To all incubation samples ^{14}C-5 HT was added to give a final radioactivity level of 0 3 µCi per ml

Histamine

10 mM Hi dihydrochloride was titrated to pH 7 with 10 mM Hi base ^{14}C Hi was added to each incubation sample to give a final radioactivity level of 0 3 µCi per ml

Sodium

30 mM sodium dihydrogenphosphate (= 30 meq Na/L) was titrated with 15 mM disodium hydrogenphosphate 30 meq Na/l) to pH 7 ^{22}NaCl was added to each incubation sample to give a final radioactivity level of 0 2 µCi per ml

Influence of pH on granule 5 HT and Hi storage

A *Depleted granules recharged with 5 HT or Hi*

Weighed samples of depleted and dried granules were stirred for 15 min at room temperature in 1 mM 5-HT solution pH 7 containing ^{14}C 5 HT or in 0 3 mM Hi solution (pH 7) containing ^{14}C Hi (for incubation fluids see above) The chosen concentrations were calculated to give 50 75 per cent saturation of the granule stores (Fig 3) After centrifugation at $3000 \times g$ for 20 min the supernatants were sucked off the radioactivity recorded and the weight of the remaining droplet measured as described above in order to allow correction for contaminating radioactivity The sediments were incubated in 1 ml of deionized water or in 1 ml of hydrochloric acid of increasing concentrations (10^{-6} to 10^{-2} M) After centrifugation at $3000 \times g$ for 20 min 200 µl of each supernatant was added to 200 µl of 1 M NaOH and 15 ml of scintillation fluid The granules were suspended in the remaining supernatant and the pH was measured After dissolution in 200 µl of 1 M NaOH the radioactivity in 200 µl of the suspension was determined by liquid scintillation spectrometry After correction for the radioactivity left in the droplet and for the release in deionized water (pH about 6 5) the percentage release of 5-HT and Hi due to increased acidity was calculated

B *Granules charged with endogenously formed ^{14}C 5 HT*

Mast cells from 15 rats were incubated for 3 h at 37 C in 10 ml of isotonic salt solution (154 mM NaCl 2 7 mM KCl 0 9 mM CaCl₂ containing 10 % v/v Sorensen phosphate [illegible] ^{14}C-5-HTP solution 0 1 mCi [illegible] for 10 min and washed with [illegible] e in order to remove extra [illegible]

The granules were isolated and dried as described above except that to avoid cation exchange, no adjustment of pH with sodium hydroxide was carried out

The dried ^{14}C-5 HT-containing granules were suspended in deionized water and aliquots were pipetted into 1 ml deionized water or HCl solution as described for *in vitro* recharged granules

Calculation of maximal uptake, dissociation constants and inhibition constants

According to the law of mass action and assuming a reaction equilibrium between the granules (G) and 5-HT, Hi or sodium (X) then



$$G + X \leftrightarrows GX \quad (1) \qquad K = \frac{(G)(X)}{(GX)}, \quad (2)$$

Via the derivation for the Michaelis Menten equation and conformation according to Lineweaver Burk the following equation is derived

$$\frac{1}{U} = \frac{K}{U_{max}} \cdot \frac{1}{(X)} + \frac{1}{U_{max}}, \quad (3)$$

where U is the uptake (mμmoles or mμeq/mg granules), U_{max} the maximal uptake, (X) the concentration of 5 HT Hi or sodium (mM meq/L) and K the dissociation constant of the

U_{max} and K were calculated (Table III)

In the case of competitive antagonism as shown in Fig 6 and 7, the inhibition constant (K_i) may be calculated from

$$K = K_{5\ HT}\left(1 + \frac{(I)}{K_i}\right) \quad (4)$$

where K is the dissociation constant for 5 HT in the presence of the competitive inhibitor I The equation describing curves b c and d in Fig 6

$$\frac{1}{U} = \left(1 + \frac{(I)}{K_i}\right) \frac{K}{U_{max}} \cdot \frac{1}{(X)} + \frac{1}{U_{max}}, \quad (5)$$

where U is the uptake of 5 HT in the presence of a competitive inhibitor is equal to equation (3) with exception that the angle coefficient will be multiplied by a factor of $\left(1 + \frac{(I)}{K_i}\right)$

By calculating the K from curve c and K_X and U_{max} from curve a and using these values in equation (5) the U may be calculated for any value of (X)

Materials

Histamine (ring 2 ^{14}C) dihydrochloride Sp act 54 mCi/mmole DL-5 hydroxytryptophan (methylene-^{14}C) Sp act 58 mCi/mmole $^{22}NaCl$ 1 mCi/mg The Radiochemical Centre Amersham England

5 hydroxytryptamine 2 ^{14}C binoxalate Sp act 18 mCi/mmole New England Nuclear Boston Mass USA

Ficoll AB Pharmacia Uppsala Sweden

Human serum albumin (free from preservatives) AB Kabi Strangnas Sweden

All other substances were obtained from usual commercial sources

Results

Contents of Hi and 5 HT in rat mast cells

The Hi and 5 HT contents found were within the limits reported by previous authors the molar ratio Hi/5 HT being 55—60 (Table I)

Distribution of Hi and 5 HT after osmotic lysis of mast cells

Isolated mast cells were washed in isotonic sucrose at 0 C in order to minimize the contamination of the suspension medium with buffer salts and then lysed in deionized water The distribution of Hi and 5 HT in coarse cell debris (400×g 15 min) in

TABLE I Content of 5 HT and Hi in rat mast cells separated by density gradient centrifugation

Conc Ficoll %	Cells/ml	µg/10⁶ mast cells		Molar ratio
		5 HT	Hi	Hi 5 HT
30	1 10⁴	0 47	16 0	55
40	2 8 10⁴	0 43	15 4	58
50	2 10⁴	0 53	19 9	60

TABLE II Distribution of 5 HT and Hi after density gradient centrifugation of osmotically lysed mast cells

Fraction	5 HT %	Hi %
Sediment 400 × g	30	50
Sediment 3000 × g	6	20
Supernatant 3000 × g	64	30

the granule fraction and in the supernatant (3000 × g, 20 min) showed considerable differences (Table II) between the two amines For discussion on this point see p 329

Influence of sodium phosphate buffer on granule amine stores

Mast cells washed in isotonic sucrose in order to minimize the contamination with salts remaining from the isolation procedure were lysed in deionized water and centrifuged at 3000 × g Aliquots of the granule containing precipitate were suspended in sodium phosphate buffers of various concentrations at pH 7 After recentrifugation at 3000 × g Hi and 5 HT were determined in precipitates and supernatants Both amines were released from the granules the higher the buffer concentration the greater the release When the sodium concentration reached 100 meq/L the granule Hi and 5 HT stores were reduced to about [illegible] 10 % respectively (Fig 2)

Storage capacity of isolated granules

Dried amine- and sodium-depleted granules were suspended in sodium , Hi- and 5 HT-solutions at pH 7 as described on p 322 Within the concentration range used —0 003—30 mM (meq/L)—the three uptake curves show similar courses, reaching an uptake of around 1000 mµmoles (mµeq)/mg granules at concentrations between 1 and 10 mM (meq)

The maximal uptake, and the dissociation constants for the sodium , Hi and 5-HT-binding were calculated from a double reciprocal plot (Table III)

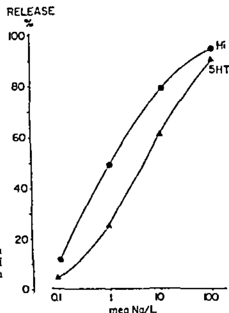


Fig 2 Release of 5-HT (▲——▲) and Hi (●——●) from granule containing sediment of osmotically lysed mast cells suspended in sodium phosphate buffer at pH 7

The effect of pH on Hi- and 5-HT-storage

If 5 HT is bound to the granule by a simple ionic linkage between the primary amino group of the ethylamine side chain and acid groups in the granules, the capacity of the granules to store the amine at pHs below 7 should depend on the dissociation of the acid groups This assumption was tested by exposing 5 HT-containing granules to media of pHs between 7 and 2 5, thus influencing the dissociation of carboxyl groups

Depleted granules were recharged with Hi or 5-HT by suspension in 0 3 mM Hi (+^{14}C Hi) and 1 mM 5-HT (+^{14}C-5-HT) respectively The amine concentrations were calculated to give a 50—75 % saturation of the granule amine stores (see Fig 3)

On acidification of the granule suspensions as described on p 322, the granule amine stores were gradually depleted, retaining only 10—15 % of their amines at pH 3—2 (Fig 4) The 5 HT depletion curve runs parallel to the Hi curve but is

TABLE III Calculated maximal uptake and K values for the granule binding of 5 HT Hi and sodium

	Maximal uptake per mg granules	K (M)
Hi	900 mμmoles	$9 \cdot 10^{-6}$
5 HT	900 mμmoles	$3 \cdot 10^{-4}$
Na	1000 mμeq	$1 \cdot 10^{-3}$

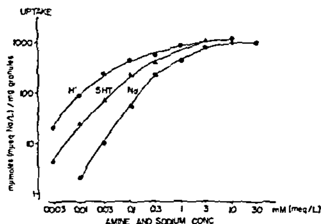


Fig 3 Uptake of 5 HT (▲—▲) Hi (●——●) and sodium (Na) (○——○) by depleted mast cell granules (pH 7, 20° C) Corrections were made for concentration reduction due to granule uptake of the amines and sodium

displaced to the right, indicating a weaker affinity of 5-HT for the ionic sites The pH sensitivity of both Hi and 5-HT-stores indicates a dependence of the storage capacity on COO groups and not on strongly acidic groups

The above studies were performed on granules recharged with 5-HT *in vitro* To meet the argument that such exogenous 5 HT might behave differently from endogenous 5 HT in its binding properties observations were also made with endogenous 5-HT Mast cells were suspended in ^{14}C-5 HTP-containing media The mast cells formed ^{14}C 5 HT and stored it in their granules (Slorach and Uvnäs 1968 When the ^{14}C-5 HT-containing granules were isolated and exposed to media of various pHs the release curve for endogenous 5 HT was found to follow very closely the corresponding curve for granules recharged with 5 HT *in vitro* (Fig 4)

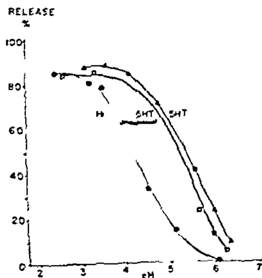


Fig 4 Influence of pH on granule 5-HT and Hi storage Depleted granules recharged in 1 mM 5-HT (▲——▲) or 0 3 mM Hi (●——●) Granules charged with ^{14}C 5 HT (□——□) by incubation of mast cells *in vitro* with ^{14}C-5-HTP endogenous 5-HT) Corrections were made for amine release due to "water effect

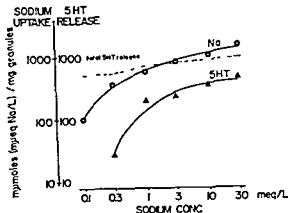


Fig 5 Release of 5 HT and uptake of sodium Depleted granules recharged in 3 mM 5 HT Sodium induced 5 HT release ▲——▲, sodium uptake O——O, total 5-HT release (due to sodium and water) - - - -

Release of 5 HT and uptake of sodium by granules

'Depleted' granules recharged with 5-HT were suspended in sodium phosphate buffers of various concentrations, all at pH 7 Two parallel series of experiments were performed, one with ^{14}C-5-HT and unlabelled sodium, the other with unlabelled 5-HT and ^{22}Na In this way the release of ^{14}C-5-HT and the uptake of ^{22}Na could be recorded in corresponding samples and the figures used for calculations of the equivalent ratio between the uptake of sodium and release of 5 HT Fig 5 shows the results of an experiment in which the granules were recharged with 5-HT by suspending them in 3 mM 5-HT, a concentration chosen to charge the granules with about 1000 mμmoles/mg (see Fig 3) Resuspension of these granules in phosphate buffers resulted in a significant uptake of sodium already at a sodium concentration of 0 1 meq/L The uptake rose with increasing sodium concentrations, reaching about 1000 mμeq/mg between sodium concentrations of 10—30 meq/L 5-HT was concomitantly released The total 5 HT release also reached about 1000 mμmoles/mg at the maximal sodium concentration used However, to judge from the release curve,

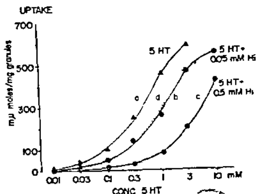


Fig 6 Competition between 5-HT and Hi for granule storage sites in depleted granules

Curve a uptake of 5-HT at pH 7

Curve b uptake of 5-HT in the presence of 0 05 mM Hi at pH 7

Curve c uptake of 5-HT in the presence of 0 5 mM Hi at pH 7

Curve d calculated uptake of 5-HT in the presence of 0 05 mM Hi calculated from curves a and c (see text)

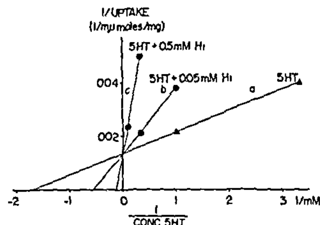


Fig 7 A double reciprocal plot of the "linear part" of curves a, b and c in Fig 6

the lowest sodium concentration used had no appreciable 5-HT releasing effect *per se*, the 5-HT release observed evidently being a "water effect". The sodium-induced 5-HT release was calculated by deducting this "spontaneous" 5-HT release from the "total" release recorded This calculated release curve ran rather parallel to the sodium uptake curve, the equivalent ratio Na-uptake/5-HT-release being about 3—4 This was the lowest ratio obtained in any experiment If the granules were recharged in 5-HT concentrations calculated to give only a partial saturation of the binding sites higher ratios were regularly obtained The interpretation of these findings will be discussed below

Competition between 5-HT and Hi for granule storage sites

Depleted granules were recharged by suspension in 0 01—10 mM 5-HT, giving a 5-HT uptake curve as seen in Fig 6 a Two other 5-HT uptake experiments were performed with identical 5-HT concentrations but in the presence of Hi, in one series 0 05 mM, in the other 0 5 mM Hi The presence of Hi caused a shift to the right of the uptake curves for 5-HT (Fig 6 b and c) A double reciprocal plot of the uptake curves illustrates the competition of 5-HT and Hi for the storage sites (Fig 7)

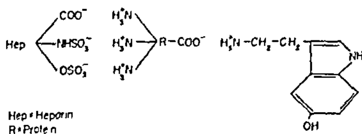


Fig 8 Assumed gross structure of the heparin protein 5 HT complex of mast cell granules at pH 7.

The dissociation constant (K) and maximal uptake (U_{max}) for 5 HT can be calculated from curve a (Fig 6) using equation (3) page 323 The dissociation constant for 5 HT in the presence of Hi (K) is calculated from curve c (Fig 6) and the inhibitory constant (K_i) is calculated from equation (4) page 323 From the values so obtained from curves a and c (with 0 5 mM Hi) curve d was calculated for 0 05 mM Hi (equation (5) page 323) As seen this calculated uptake curve for 5 HT coincides with the experimentally found uptake curve b (with 0 05 mH Hi)

Discussion

From previous studies on the mechanism of Hi storage in isolated mast cell granules it was concluded that these granules had storage properties like a weak cation exchange resin (Uvnas *et al* 1970) The influence of pH on the uptake and release of sodium and Hi quantitative studies on the granule storage capacity and titration studies on the granules convinced us that the storage sites were carboxyl groups in the heparin protein complex which forms the matrix of the granules The findings further indicated that the binding carboxyls belonged to the protein part of the heparin protein complex

The present 5 HT studies have further strengthened our belief that the mast cell granule amine storage and relase mechanisms are based on a simple cation exchange principle

The pH dependence of the granule 5 HT storage suggested it to depend on available dissociated carboxyl groups When exposed to sodium the granules released their 5 HT and took up sodium in exchange When the granules were exposed simultaneously to 5 HT and Hi a competition for uptake occurred indicating that the two amines were competing for identical storage sites

Our results seem to us to warrant the conclusion that 5 HT like Hi and sodium ions is stored in ionic linkage to COO groups in the granule matrix possibly to protein carboxyls in the heparin protein complex For further argumentation concerning the localization of the binding COO groups see Uvnas *et al* (1970) The dissociation constant of the 5 HT granule binding was calculated to be 3×10^{-4} *i e* greater than that for Hi (9×10^{-5}) but smaller than that for Na (1×10^{-3}) This intermediate position of the affinity of 5 HT to the binding sites is reflected in the water effect on the granule stores 5 HT which has a smaller affinity than Hi leaks out from granules suspended in water to a much greater extent than Hi does (see Table III and Fig 5) Accordingly sodium ions which have the lowest affinity to the granule sites are easily removed by washing in water (Uvnas *et al* 1970)

The wash out effect of 5 HT also explains the high equivalent ratio between sodium uptake and 5 HT release in granules When granules had been suspended in concentrations of 5 HT calculated to give a maximal uptake of the amine (1000 mμmoles/mg granules) the suspension of these granules in NaCl-containing media should be expected to result in an equivalent exchange between Na ions and 5 HT However the uptake of sodium ions was found to be 3—4 times higher than the

corresponding release of 5 HT (Fig 5) The explanation for this high ratio (Na/5 HT) is simply the fact that the recharged granules when resuspended in water lose 60—70 % of their 5 HT (see Fig 5) This means that out of the recharged 1000 mμeq of COO groups per mg granules only 300—400 retain their 5 HT The other COO groups will become occupied by H ions When such granules are then suspended in sodium phosphate buffers of sufficient concentrations, sodium will occupy all the COO groups, not only the 5 HT' but also the H binding sites The equivalent ratio between Na uptake and 5 HT release will consequently be greater than 1 The less saturated the stores are with 5 HT, in other words the more thoroughly the granules are washed or the lower the 5 HT concentration in which they have been incubated the higher will the ratio be between sodium taken up and 5 HT released

In our view Hi release caused by degranulating agents is due to a cation exchange occurring when the mast cell granules become exposed to the extracellular cation containing medium (Uvnas 1964 Uvnas and Thon 1965 Åborg and Uvnas 1968 Nosal Slorach and Uvnas 1970) Since during degranulation the granules lose their protective membrane exposure to cation containing media will lead to an instantaneous amine release Since 5 HT seems to be stored at ionic sites identical with those which bind Hi 5 HT release from mast cells can also be assumed to be due to an ion exchange following degranulation The results of Moran Uvnas and Westerholm (1962) are in agreement with this view They observed

1) that when mast cells were treated with compound 48/80 the time courses of release of the two amines were almost identical
2) similar dose response relationships
3) lack of selective release of either amine alone by the releasing agents tested
4 similar pH dependence
5 similar sensitivity to inhibitors etc

The similarity of the storage and release mechanisms for 5 HT and Hi raises the question of common or separate subcellular stores for the two amines All rat mast cells show fluorescence reactions characteristic for both Hi and 5 HT, but it is not known whether the amines also share the same granules The molar ratio in rat mast cells between Hi and 5 HT was found to be about 60 1 To our knowledge the fluorescence characteristics of isolated granules have not been studied—probably due to technical difficulties—since the granules are apt to lose most of their amines especially 5 HT during the necessary preparative procedures However our results do not contradict the idea that both Hi and 5-HT are localized to the same granules

The suggested binding of 5 HT to heparin (Keller 1958) deserves some comment As is the case with Hi the theory about the binding of 5 HT to granule heparin is based solely on the fact that *in vitro* heparin has been found to form a complex with this amine The heparin histamine complex has especially attracted much interest due to its stability in water even in rather acid milieu (pH 3—2) and its lability in the presence of salts

In a previous paper (Uvnas *et al* 1970) we have argued against the tenability of

the hypothesis advocating the heparin histamine complex as being the form in which Hi is stored Similar arguments can be used against the corresponding ideas about 5 HT storage. The *in vitro* 5 HT heparin complex is supposed to be formed by the strong attractive forces of the sulphated groups of the heparin and the amine groups of the 5 HT But as pointed out previously (Uvnäs *et al* 1970), in the granules the heparin is not free but occurs as a component of a heparin protein complex In this complex the acid groups of the heparin are probably firmly bound to amino (and imino) groups in the strongly basic polypeptide which forms the protein part of the granule complex Consequently there will be no sulphated acid heparin groups available for the binding of 5 HT Furthermore Sanyal and West (1959) found that 5 HT failed to form a complex with heparin *in vitro*

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Acta physiol. scand. 1972. 84. 332—337

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The Influence of the Crowding Phenomenon on the Oxygen Consumption of Blood Cells as Determined by the Cartesian Diver Technique

By

Ingebrigt Talstad

Received 7 September 1970

Abstract

Talstad I *The influence of the crowding phenomenon on the oxygen consumption of blood cells as determined by the Cartesian diver technique* Acta physiol scand 1972 84 332—337

The tendency of decrease in oxygen (O_2)-consumption per cell with increase in cell concentration during measurements (crowding phenomenon) was studied with the Cartesian diver technique The theoretical linear relationship between O_2 consumption and the number of cells was found for polymorphonuclear leukocytes (PMN) lymphocytes and platelets with the standard divers the capillary type of divers giving the same results as standard divers However marked crowding phenomenon occurred at high numbers of PMN lymphocytes or platelets per diver An explanation for the crowding phenomenon is probably deficient O_2-equilibration during measurements and not a peculiar phenomenon of PMN or lymphocytes as has so far been suggested

The crowding phenomenon *i e* the tendency of decrease in oxygen (O_2)-consumption per cell with increase in cell-concentration during measurements has repeatedly been shown for leukocytes (Hartmann 1952 Luganova and Seits 1958 McKinney *et al* 1953 Soffer and Wintrobe 1932) and lymphocytes (Hedeskov and Esmann 1966) The explanation for the crowding phenomenon has not been established but has been considered a peculiar phenomenon of leukocytes

The possibility that the crowding phenomenon might be related to the technique in measurement, was the object of the present study It was decided to examine whether crowding phenomenon might be demonstrated for other cells than leukocytes *e g* platelets

Materials and methods

P[illegible] lear leukocytes (PMN lymphocytes and platelets were separated [illegible] d 1970 a) [illegible] 2 mg/ml [illegible] filling the

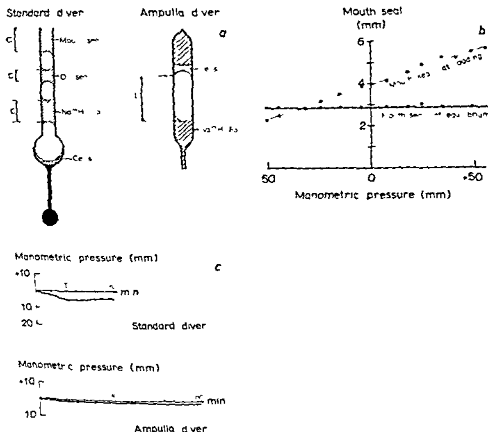


Fig 1 (a) The standard and ampulla standard diver are shown (b) The relation between the length of mouth seal at loading and at equilibrium position of the same diver (c) The change with time in manometric pressure at equilibrium position of 10 control divers (± 2 S.D)

divers ("before counts) After completing the O_2-measurements the divers were rinsed into a beaker containing diluting fluid for counting (after counts) using an electronic particle counter (Celloscope 101 Lars Ljungberg Sweden) (Talstad 1971 b)

The Cartesian diver technique The apparatus was essentially that described by Holter (1943) The temper … n Ultra term (Heto …)° C

Two type … (1943) and a new … 1966 but of the same size as standard divers to facilitate pipetting of cell samples This ampulla standard diver (Fig 1 a) was prepared from thin walled glass capillaires of ~ 1 mm internal diameter and ~20 mm in length which were sealed into a conical shape at one end by a small flame The capillary part of the diver was drawn out after the cell had been pipetted into the conical part of the diver

The air volume of standard divers was calculated from the equatio

where the

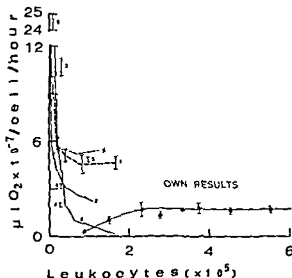


Fig 2 The O^2-consumption of PMN measured with standard divers (Own results) is compared to results given in the literature derived from measurements with Warburg apparatus The numbers 1—6 correspond to references by Hartmann 1952, Hedeskov

a measuring ocular The size of the mou... independent of the loading size (Fig 1 ... to the volume of Hg delivered by that ... simple since V_t v and m were constan... V_{air} simplified the technique, avoiding ... neck seals (Holter 1943) Simple drawn out glass capillaries were satisfactory for all pipettings. Care was taken to get the diver to float at a suitable manometric pressure (+5 to −20 cm Brodie fluid) major adjustments of the divers weight was done by removing or adding glass to the tail minor adjustments by pipetting air between the mouth and oil seal

The air volume of the ampulla standard divers was calculated from the equation

$$V_{air} = l\ v + \pi\ (d/2)^3/3 = l\ v + m \sim l\ v \qquad (II)$$

where the length of the air column (l) was measured from the negative film of the diver and a mm-scale using a magnifying apparatus (Fig 1 a) The volume/mm of the diver (v) was determined after completing the O_2 measurements, by weighing with Hg after the diver had been cut and cleaned

Fig 1 c shows that the control divers did not differ significantly from the zero line during the measurements

Calculation of O^2 consumption (ΔV, μl 0^2/hour at NTP

$$\Delta V = V_{air}\ \ \Delta P\ \ 60\ \ 273/10\,000\ \ t\ \ 310 \qquad (III)$$

where V_{air} is in μl and ΔP is the pressure change in mm Brodie fluid (10 000 mm = 1 atm.) in t min

The O_2-consumption/10^{10}cells/hour is obtained by multiplying ΔV with $10^{10}/C$ where C represents cells/diver

Statistical calculations Means, standard deviations coefficients of correlations and regression equations were calculated

Results

Fig 2 shows the crowding phenomenon of PMN and lymphocytes measured by different authors with Warburg apparatus compared to our own results measured with standard divers

Fig 3 a, b show that the theoretical linear relationship between O_2-consumption and platelet number was approached with standard divers when related to the

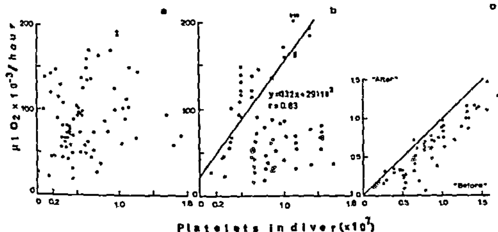


Fig 3 The relation between O_2-consumption and platelet number per diver based on "after" (a) and "before (b) counts, standard ( ) and ampulla standard divers (o) were used (c) shows the relation between the "before' and "after" platelet counts from the same diver

'before' but not the "after" counts The marked scattering of the results when related to the "after" counts was probably due to aggregation of still viable platelets

Fig 4 a, b show small differences between the O_2-consumption calculated from the 'before" and "after" counts of PMN However, markedly reduced values (hereafter referred to as "dropped values") might occur if related to the 'before" counts (Fig 4 b) probably because the PMN were particularly sensitive to injury This phenomenon did not occur with platelets (Fig 3 b) Avoiding "dropped values" the theoretical curve through the origin was approached (Fig 4 b)

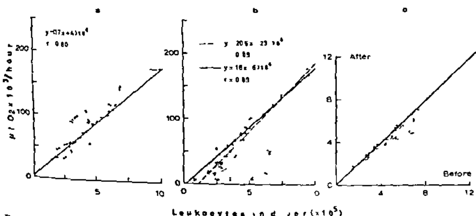


Fig 4 The relation between O_2-consumption and number of PMN per diver based on the "after (a) and "before (b) counts "Dropped values see text) were included in the line but excluded in the —— line of (b standard and ampulla standard divers (o) were used (c) shows the relation between the before and after counts of PMN from the same diver

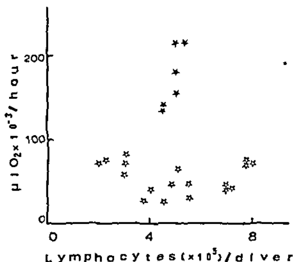


Fig 5 The relation between O_2 consumption and number of lymphocytes per diver, standard (★) and ampulla standard divers (☆) were used

Fig 3 b, 4 b and 5 show that the O_2-consumption obtained with the ampulla standard divers approached those of standard divers when cell numbers were low, but were markedly depressed at high number of platelets, PMN or lymphocytes per diver

Discussion

In our experiments both standard and ampulla standard divers were loaded with cells beyond the limits ordinarily advisable in order to test our hypothesis regarding the crowding phenomenon In ampulla standard divers, the relatively more compact cell layer than in standard divers might decrease the O_2-diffusion (Umbreit, Burris and Stauffer 1964) However this does not explain the reduction in O_2-consumption by platelets as these did not form a compact cell layer, but sedimented during measurements Incomplete O_2-equilibration in the ampulla standard divers probably explains the crowding phenomenon when high numbers of platelets, PMN or lymphocytes are present With standard divers no crowding phenomenon occurred during measurements even at high cell numbers per diver The standard divers are outstanding respirometers in facilitating the O_2 diffusion due to the small depth and large surface area available to the cell suspension No difference is found between standard and ampulla standard divers when overloading is avoided (Talstad 1971 c) The relatively high O_2 consumption and the ease with which PMN or

The results presented support our suggestion that the crowding phenomenon is related to the technique of measurement and not a peculiar phenomenon of PMN or lymphocytes as has been supposed

I am indebted to Professor Macfadyen at the Mols Laboratory Denmark for introducing me to the standard diver technique and to Professor Zeuthen Carlsbergsfondets Biologiske Institut Copenhagen for valuable advice The work was supported by the Norwegian Research Council of Science and Humanities.

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Chloride Transport across Isolated Frog Skin

By

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Abstract

Kristensen P *Chloride transport across isolated frog skin* Acta physiol scand 1972 84 338—346

A net inward transport of chloride was found to occur in isolated skins of *Rana temporia* This transport was not dependent on sodium transport and was inhibited by diamox thiocyanate and bromide The active chloride transport was not affected by changes in the transepithelial potential difference On the other hand the curve describing efflux as a function of potential difference was found to have a shape resembling the shape of the Goldman equation

Graphical determination of apparent pools showed the influx pool to be about six times greater than the efflux pool Diamox had no effect on efflux pool size but reduces the apparent influx pool so that it becomes about equal to the efflux pool This means that at least part of the paths for active and passive chloride movements are different

A working hypothesis is put forward according to which chloride is actively transported across the outward facing membrane of the outermost living cell layer of the frog skin epithelium and the main part of the passive flux occurs across the tight seals and via the interspace system

Krogh (1937) and Jorgensen Levi and Zerahn (1954) found reasons to believe that in intact frogs chloride is actively transported from the mucosal to the serosal side of the frog skin A similar behaviour of chloride was observed by Martin and Curran (1966) on isolated skins of *Rana pipiens* and *Rana esculenta* when the skins were bathed in Ringer s with low chloride concentration The fluxes were too small to cause a discrepancy between measured sodium net flux and short circuit current A much larger chloride transport was observed in the skins of *Leptodactylus ocellatus* studied by Zadunaisky Candia and Chiarandini (1963) but in this species chloride transport was inhibited by ouabain

The aim of the present work is to characterize in greater detail the chloride transporting system of the isolated skin of *Rana temporaria* A working hypothesis is discussed including the possible localization of the pump to the outward facing membrane of the outermost living cell layer the possibility of chloride combining with some membrane constituent to form an electrically neutral complex, which

TABLE I Fluxes of chloride measured on short-circuited frog skins bathed in sulphate Ringer's containing 1 mM chloride Skin area 7 cm² Fluxes are given in neq h

Influx	Efflux	Flux Ratio
187	9 4	19 9
230	19 2	12 1
260	5 1	51 0
23	4.2	5 5
8 7	3 1	2 8
141	4 3	32 8
258	17 5	14 7
2?5	16 4	13 7
139	21 3	6 5
170	21 0	8 1
35?	10 4	34 1
425	13 0	32 6

crosses the membrane, and the paths for active and passive chloride movements across the skin under circumstances of low chloride concentration (1—2 mM) in the bathing solutions

Methods

The frogs were kept in tap water at 4° C and decapitated immediately before the experiments The skins were dissected off divided into two laterally symmetrical halves and mounted between two perspex half chambers The areas of the skins were in all experiments 7 cm² Both sides of the skins were in contact with 25 ml of a chloride containing sulphate Ringer's 56 5 mM Na_2SO_4 2 4 mM $NaHCO_3$ 2 0 mM K_2SO_4 1 0 mM $CaSO_4$ and 1 0 mM NaCl The skins were washed in the chambers two times for periods of 20 min before the flux measurements were performed The fluxes were measured with ^{36}Cl luC being added to the outside bathing solution of one skin half and to the inside solution of the other skin half This increases the chloride concentration which finally in all experiments was 1 34 mM Samples of 1 ml were withdrawn and counted to an accuracy of 2 per cen or better Packard Liquid Scintillation Spectrometer) Samples were replaced with an equal amount of Ringer's having the same composition as the sample and correction for this dilution included in the calculations All flux values reported are averages of three or four consecutive periods each of 20 or 30 min Only experiments in which fluxes were constant during the time of measurement are included in this report.

Results

The presence of an active chloride transport in skins of *Rana temporar*a is clearly shown by Table I in which chloride fluxes are measured on [illegible] skins bathed on both sides in sulphate Ringer's containing 1 [illegible] hloride The magnitude of this transport is small (average about [illegible] mpared to the normally measured sodium transport (about 10 eq [illegible] a not be expected to give any descrepancy between short-circuit curr[illegible] sodium flux [illegible] was the case in the experiments reported by Zadu[illegible] Candia and Ch[illegible]andini (1963) on *Leptodactylus ocellatus* But it corresponds well with chloride transport rates in skins of of other *Rana* species (Martin and Curran 1966)

TABLE II. The effect of various compounds on chloride net transport across the isolated, short-circuited frog skin Area of the skin 7 cm²

Compound	Concentration mol/l	Chloride net flux neq/h	
		Control period	*Test* period
Dinitrophenol	2×10^{-4}	21.3	8 0
Dinitrophenol	2×10^{-4}	292 6	12 9
Dinitrophenol	2×10^{-4}	19 8	—0 1
Dinitrophenol	2×10^{-4}	18 0	2 8
Dinitrophenol	2×10^{-4}	50 5	5 8
KCN	4×10^{-4}	143 2	19 2
Diamox	$0\ 8 \times 10^{-3}$	230 0	0 0
Diamox	$0\ 8 \times 10^{-3}$	181 5	4 5
NaBr	2×10^{-3}	118 0	27 4
NaBr	2×10^{-2}	208 6	61 6
NaBr	5×10^{-3}	412 0	116 0
NaBr	10^{-2}	55 0	11 0
NaSCN	10^{-3}	345 0	58 0
NaSCN	10^{-3}	149 0	11 8
Ouabain	10^{-4}	130 0	126 0
Oubain	10^{-4}	20 4	21 3

TABLE III Chloride fluxes measured in short circuited skins in the presence of ouabain (10^{-4} M in the inside sol) and amiloride (5×10^{-5} M in the outside solution) Skin area 7 cm² Fluxes are given in neq/h

Influx	Efflux	Net flux
290 6	19 3	271 3
151 9	45 5	106 4
74 7	39 8	34 9
149 6	63 0	86 6

TABLE IV *The effect of sodium free solutions on the outside on chloride fluxes in short circuited* frog skins Skin area 7 cm² The isotope was added as HCl The fluxes are given in neq/h

	Composition of bathing solutions		Influx	Efflux	Net flux
	Inside	Outside			
Contr	Na_2SO_4-R	Na_2SO_4-R	228 1	3 5	224 6
Exp	Na_2SO_4-R	$KHCO_3$, 2 4 mM	190 8	4 4	186 4
Contr	Na_2SO_4-R	Na_2SO_4 R	210 0	24 0	186 0
Exp	Na_2SO_4-R	$KHCO_3$, 2 4 mM	155 0	12 0	143 0
Contr.	Na_2SO_4-R	Na_2SO_4-R	323 9	18 9	305 0
Exp.	Na_2SO_4-R	$KHCO_3$, 2 4 mM	259 5	2 5	257 0
Contr.	Na_2SO_4-R	Na_2SO_4-R	130 1	6 0	124 1
Exp	Na_2SO_4-R	$KHCO_3$, 2 4 mM	117 9	1 6	116 3

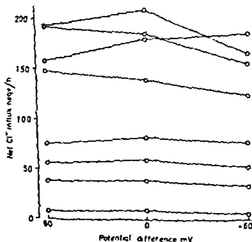


Fig 1

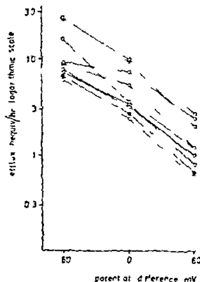


Fig 2

Fig 1 Net chloride influx plotted against transepithelial potential difference Points reprent ing individual experiments are connected by lines

Fig 2 Open circles Efflux of chloride as a function of transepithelial potential difference Filled circles Theoretical values obtained by the Goldman equation Individual experiments are connected by lines.

The experiments of Table II showing the inhibitory effect of dinitrophenol and KCN indicate that the assymmetry in fluxes is dependent on metabolic energy, supporting the view that chloride is actively transported

To test if the small chloride transport is a result af gradients in the skin formed by the much larger sodium transport the effect of ouabain was tested Table II shows this compound to have no effect and the presence of an active transport of chloride under the simultaneous presence of ouabain and amiloride (an inhibitor of sodium transport, Baba *et al* 1968) supports the view that chloride transport does not occur by a coupling to sodium transport (Table III) A slight inhibition (average 16 per cent) of chloride transport is seen when the outside bathing solution is made sodium free (Table IV)

In order to investigate the dependence of chloride transport on the transepithelial potential difference influxes and effluxes were measured as usual on paired skin halves at PD values of +60, 0 and −60 mV (inside positive The net fluxes were calculated from the measured influxes by subtraction of the passive influxes which were calculated from the measured effluxes by Ussing's flux ratio equation (Ussing 1949) The results of these experiments are seen in Fig 1 showing that the active chloride transport is not changing with the potential difference across the skin

TABLE V Apparent chloride pools and their lag times in isolated short-circuited frog skins area 7 cm². The final concentration of diamox was 0 8 × 10⁻³ M

	Influx				Efflux			
	Lag time min		Apparent pool nequiv		Lag time min		Apparent pool nequiv	
Exp no	Contr	Diamox	Contr	Diamox	Contr	Diamox	Contr	Diamox
1	3 5	12 0	1 8	0 9	11 0	11 0	0 5	—
2	3 0	6 5	4 0	1 3	10 0	10 0	0 6	0 5
3	11 3	12 5	1 7	0 7	18 5	12 5	0 6	0 7
4	6 5	6 0	15 0	4 5	5 0	6 5	3 0	4 0
5	5 0	19 0	7 5	5 6	20 0	31 0	0 6	3 3
6	4 5	5 5	11 5	2 0	15 5	12 5	0 9	1 7

The method used to obtain values for the passive influxes is however not allowed if the passive flux is occurring by exchange diffusion or if the efflux occurs as a result of partial reversibility of the pump mechanism In these two cases one would expect to obtain effluxes which were not dependent on the potential difference across the skin Fig 2 shows however that the efflux does vary with the potential [di]ference The relationship may be compared to the Goldman equation (Goldman [19]43—44 and Levi and Ussing 1949) which describes the flux of an ion across [a] single membrane The points representing the Goldman equation in Fig 2 have [be]en calculated with adequate arbitrary constants in order to obtain values comparable to those measured in the experiments The experiments thus indicate that the major part of the passive chloride fluxes occurs by a mechanism different from exchange diffusion or backflux through the pump The similarity to the shape of the Goldman equation opens the possibility that chloride moving from the inside to the outside only passes one membrane

In order to make further conclusions it is necessary to have information about the possible localization of the chloride transporting system An attempt was therefore made to estimate the apparent pools of chloride for influx and efflux This was done by following the appearance of isotope on one side of the skin after addition to the other The results were treated graphically (see Andersen and Zerahn 1963 or Harvey and Zerahn 1969) The amounts of chloride accumulating were plotted against time and the apparent pool size determined as the vertical distance between the steady state line and a line parallel to this through zero time The horizontal distance between these two lines the "lag time" (Harvey and Zerahn 1969), is a measure of the equilibration speed of the apparent pool Table V shows that the influx "pool" is about six times greater than the efflux "pool" In most experiments the *lag time is shorter for the influx pool* Diamox has no significant effect on efflux pool size or lag time

Discussion

The experiments in this study clearly supports earlier conclusions (Krogh 1937, Jørgensen Levi and Zerahn 1954, and Martin and Curran 1966) that chloride is actively transported from the mucosal to the serosal bathing medium in the skins of *Rana* species, although the rates of this transport is very much smaller than the rates of sodium transport in that tissue

The fact that chloride transport is not inhibited by ouabain, amiloride, and only slightly inhibited by the absence of sodium in the outside bathing solution classifies it as a primary active transport system This is further supported by the fact that ions (Br, SCN) which inhibit chloride transport have no effect on active sodium transport, but may be thought to interfere directly with a chloride binding site because of their chemical similarity to chloride The lack of an effect of ouabain is contrary to the effect of this compound on chloride transport in *Leptodactylus ocellatus* (Zadunaisky *et al* 1963) and frog stomach (Cooperstein 1959)

Although the $KHCO_3$ solution used in Table IV is prepared nominally free of sodium it will not be so during the whole experiment due to the flux of sodium from the inside The small inhibition seen when sodium is removed from the outside solution may therefore indicate that sodium in very small concentrations is of significance for chloride transport But as the chloride transport was constant during the time of measurement while the sodium concentration must have increased it seems safe to conclude that these experiments show that chloride transport does not depend on the presence of sodium in the outside bathing medium On the other hand it cannot yet be excluded that a small fraction of the active chloride transport occurs by a different mechanism which is dependent on sodium

The fact that diamox (acetazoleamide) inhibits chloride transport points to a connection between this transport and carbonic anhydrase and therefore possibly to a link to bicarbonate transport The problem is under investigation but the experiments with dinitrophenol indicate that a bicarbonate gradient is not the only driving force If this was the case dinitrophenol might be expected to cause an increased transport of chloride

The dependence of chloride transport on the transepithelial potential difference (PD) was investigated to decide whether chloride is transported across the membrane as the free ion or as a complex with some membrane constituent (carrier) The active transport was found to be independent on PD The interpretation is however not simple If it is assumed that the outermost living cell layer is responsible for the transport, there are two possibilities for the localization of the pump The outward facing membrane and the membrane facing the interspace system It is also reasonable to assume that the amount of chloride handled by the pump is dependent on the chloride concentration in direct contact with the pump If this is located in the outward facing membrane the concentration of chloride determining interaction with the pump is the outside chloride concentration which does not change with PD In this case the result indicates that chloride combines with a

carrier forming an electrically neutral complex, which is then moved across the membrane If, on the other hand the pump is located in the inward facing membrane of the same cell layer the chloride concentration in the cells will determine interaction with the pump mechanism This concentration is dependent on the potential difference between the cell and the solution in which it is bathed In the case of an epithelium it may be considered to be in equilibrium partly with the inside and partly with the outside bathing solutions Exact calculations are not feasable because they would have to include unknown parameters like the ratio between chloride permeabilities of the two membranes and the potential jump across the inward facing membrane

If chloride is transported across the inner membrane as the ion or as an ionic complex the transport rate should increase when we go from the short circuited state to a PD value of 60 mV (inside positive) If a considerable part of the PD change is happening over the inner membrane the chloride concentration in the cells may be reduced This would mask the expected increase in transport because less chloride would now be available for the pump From the microelectrode studies of Ussing and Windhager (1964) it may however be seen that in going from the short circuited state to the open state the whole PD change does not occur exclusively across one of these membranes It is therefore not probable that a full masking as outlined above should occur

If we imagine the frog skin to be a three compartment system made up of the outside bathing solution the cellular phase and the inside bathing solution it is quite clear that the apparent influx and efflux pools ought to be of equal size That this is not the case (Table V) indicates that at least parts of the two paths are separate Looking at the relative sizes of the two apparent pools the simplest assumption would be that the active transport of chloride happens via the cells whereas the major part of the passive flux occurs through the interspace system When the influx pool is measured in the presence of diamox it is seen in Table V that this approaches the size of the efflux pool This is exactly what would be expected if the pump mechanism is located in the outward facing membrane and if this membrane is relatively tight to passive movements of chloride If the pump mechanism were located in the inside facing membrane of the outer epithelial cells the influx pool ought not to be decreased by diamox So if this compound really interacts with the active step in chloride transport it may be concluded that this step is located in the outward facing membranes of the outermost living cell layer in the frog skin

Now going back to the effect of PD on chloride transport it has been shown by Ussing and Windhager (1964) that the cells are about 20 mV negative with respect to both bathing solutions in short-circuited skins In open skins the first recordings obtained when the microelectrode is moved into the skin indicate that the first living cell layer is positive with respect to the outside This means that by changing the PD across the skin we also change the PD across the outward facing membrane By doing this we do not affect the rate of chloride active transport and provided

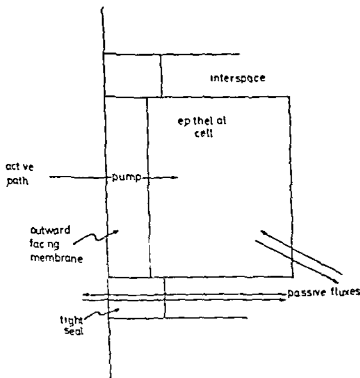


Fig 3 A schematic drawing of the outermost living cell layer indicating the working hypothesis outlined in the discussion

that the pump is located here it may be concluded that chloride is transported in combination with a carrier as an apparently electrically neutral complex The calculations of passive influxes from measured outfluxes by Ussing s (1949) flux ratio equation are justified by the dependence of efflux on PD (Fig 2) The close similarity of the experimental results with the shape of the Goldman equation is in agreement with the view that only one barrier is of significance in determining the passive chloride fluxes However other possibilities cannot be ruled out until more parameters are available for a more concise use of the equation The mentioned barrier might be the tight seals between the outermost living cells

Fig 3 is a schematic drawing of the major features f the working hypothesis outlined above It is not possible from the present material to judge about the physiological significance of this chloride transport But it may be valuable in helping us to understand how chloride passes biological membranes

The expert technical assistance of Miss Elsebeth Sander is gratel

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Circulatory Control via Vagal Afferents

I Adjustment of heart rate to variations of blood volume in the rat

By

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Abstract

CASTENFORS, J and T SJOSTRAND *Circulatory control via vagal afferents I* Adjustment of heart rate to variations of blood volume in the rat Acta physiol scand 1972 84 347—354

On withdrawal of 20—35 % of the blood volume the heart rate may increase decrease or remain unchanged The effect has been found to be dependent on animal strain anesthesia and the autonomic balance In Sprague-Daley rats a lowering of the heart rate has been practically constantly observed sometimes after an initial rise Bilateral vagotomy eliminates the reduction of heart rate but does not obviously influence the rise Atropine inhibits the bradycardia largely and propranolol the tachycardia. On administration of both atropine and propranolol both the lowering and the rise of heart rate are entirely eliminated Ligation of the common external and internal carotid arteries eliminates the bradycardia. Bilateral exclusion of afferents via the communicating branch between the superior and inferior laryngeal nerves usually eliminates the bradycardia.

It is presumed that in the rat the heart rate response to hemorrhage is determined by antagonistically acting receptor mechanisms which affect the autonomic tone on the heart one in a vagotonic and the other in a sympathicotonic direction The afferents mediating the bradycardia run entirely or for the most part via the recurrent nerve and the laryngeal communicans to the cranial part of the cervical vagus

According to current notions the adjustment of the heart rate to variations of the blood volume takes place mainly via baroreceptors on the arterial side among which may be reckoned also the synergistically acting stretch receptors in the heart A lowering of arterial pressure on sudden reduction of the blood volume reduces the discharges in the buffer nerves with a decrease in the vagal depresssion of the heart as a result which leads to a rise of the heart rate An increase of blood volume has the reverse effect At a given heart rate and vascular resistance however the arterial pressure is determined by the stroke volume of the heart *i e* by the end diastolic pressure of the left heart which in turn is determined by the blood volume in the pulmonary veins Primarily accordingly it is variations of the pulmonary blood volume that elicit the adjustment The adaptation of the heart rate occurs also on variations of the blood distribution between thorax and the remainder

of the body, with unchanged total blood volume, e g on changes of the body position The blood volume in the pulmonary veins may thus be ascribed a regulative significance for the circulation (Sjostrand 1953), as also assumed for the blood volume (Gauer and Henry 1963)

Several observations, however, indicate that this interpretation is incomplete After venesection of 300—900 ml on healthy subjects the circulation was found despite lowered pressure in the right atrium to be well compensated at unchanged heart rate (Warren *et al* 1945) In some subjects who showed initial signs of syncope the heart rate fell Syncope on change from supine to standing position is also usually preceded by a slowing of the heart rate The same has been found in patients with dumping syndrome in whom a lowering of the pressure in the pulmonary artery has been recorded (Castenfors Ekelund and Holmgren 1962) In the cat a lowered heart rate on rapid loss of blood has been found under certain circumstances (Öberg and White 1970) The reverse conditions that an increase of the blood volume leads to a rise of heart rate has been observed in man on several occasions (Warren *et al* 1948 Thomasson 1959 Engstedt *et al* 1967) The demonstration of the Bainbridge reflex shows that under certain circumstances this occurs also in animals (see Heymans and Neil 1958) In the dog the heart rate was also found to vary irrespective of the thoracic blood volume within wide limits (Langrehr and Kramer 1960)

In investigations of the effect of hemorrhage in the rat we have observed that most animals show bradycardia i e the opposite reaction to that expected The observation has been subjected to a more detailed analysis from which it appears that the lowering of the heart rate is mediated by afferents which run via the communicating branch between the recurrent and superior laryngeal nerves (the laryngeal communicans) to the cranial part of the cervical vagus It appears also if the effect is due to a mechanism which acts antagonistically to the baro and stretch receptors on the high pressure side

Material and Methods

Investigations have been made on rats of Sprague Dawley strain (S D) and on conventional rats originated from the Swedish germ free strain S G S) reared according to [illegible]ustafsson (1948) The Sprague Dawley rats were reared under specific pathogen free (S P F) conditions and in the ordinary way Their weight varied between 200 and 350 g

Blood pressure was recorded with an Elema differential transformer transducer (E M T 490 A) via catheters inserted into the carotid artery or the femoral artery on one side Blood [illegible] ter inserted into one of the femoral arteries [illegible] blood pressure record or from ECG fed through [illegible] 5 s Schön [illegible] The recording instruments were [illegible]

Results

On letting of blood from rats anesthetized with pentobarbital (5—8 mg/100 g b w) some animals respond by a rise others by a *lowering of the heart rate* (see Fig 1 and 2) Other rats show an initial rise which changes to a lowering of the heart rate

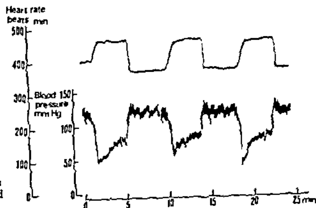


Fig 1 Heart rate response to loss and reinfusion of 25 % of blood volume in a S G S rat.

(Fig 5 and 6) Sometimes the heart rate remains unchanged On reinfusions of the blood the heart rate is usually restored, first rapidly, then more slowly The arterial pressure falls, obviously irrespective of the heart rate Variations of the heart rate proved to be dependent on the animal strain, anesthesia, the volume of blood loss and the autonomic balance

Variations with strain

S G S animals (around 10 examined) have always shown a rise of heart rate at a blood loss of 20—35 % of the calculated blood volume, and a return to normal on reinfusions (Fig 1)

S D rats have usually shown a lowering of heart rate at a blood loss of 25—35 % of the total blood volume (Fig 2 and Table I) In some animals this reaction starts with a rise of heart rate (Fig 5 and 6) The reaction has been the same in animals reared under S P F and under ordinary conditions On a minor hemorrhage some rats may respond by a rise of heart rate, and at a very high blood loss with very low arterial pressure a rise of heart rate occurs

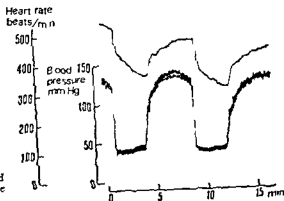


Fig 2 Heart rate response to loss and reinfusion of 25 % of blood volume in a S D rat

TABLE I Heart rate before and after loss of about 25 % of calculated blood volume

Number of rats	Conditions	Immediately before bleeding	P	Immediately after bleeding	P	30 min after bleeding
51	control	439 ±7	< 0.001	329 ±9	< 0.001	374 ±10
30	vagotomy bilat	436 ±7	—	436 ±8	—	432 ±8
5	exclusion[2] of carotid sinus bilat	415 ±11	—	433 ±26	—	416 ±21
5	sup laryngeal nerve cut bilat	420 ±17	—	418 ±15	—	427 ±6

[1] $\bar{x}$ and SE are given
[2] ligation of the common, external and internal carotid arteries

The lowering of blood pressure has to a large extent been equally pronounced in S G S and in S D rats. The blood pressure first falls rapidly and then more slowly. The same applies to the return on reinfusion. The effect both on heart rate and on blood pressure varies fairly considerably in different animals for corresponding losses of blood.

The rise or fall of heart rate persists for a long time after hemorrhage. A minor increase, however, may be found in S D rats after 1/2 h (Table I).

Effect of anesthesia

A comparison has been made between pentobarbital and ether narcosis. One S D rat was anesthetized with chloralose i p.

S D rats were anesthetized first with ether and then with pentobarbital. On bloodletting during ether narcosis there was a rise of the heart rate, but when ether was replaced by pentobarbital, a renewed blood loss led to the usual depression. The chloralose anesthetized rat responded by a lowering of heart rate.

Within fairly wide limits the reaction in S D rats seems to be independent of the depth of anesthesia. However, on one animal awakening out of pentobarbital narcosis both heart rate and blood pressure were largely unchanged on hemorrhage despite the usual depression after renewed administration of pentobarbital.

The effect of exclusion of the carotid baroreceptors

The carotid sinus and carotid body were isolated in S D rats by ligation of the common, external and internal carotid arteries. As appears from Table I, these rats showed an unchanged heart rate after hemorrhage. In a few animals the occlusion of the common carotid artery bilaterally was found to inhibit to a large extent the bradycardia (Fig 3). Occlusion of the carotid artery unilaterally on the other hand had no effect. In S G S rats occlusion of the bilateral carotid arteries does not appear to have had any definite effect on heart rate after hemorrhage.

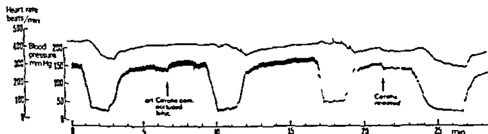


Fig 3 Heart rate response to loss and reinfusion of 25 % of blood volume before, during and after occlusion of the carotid arteries in a S.D rat.

Fig 4 Heart rate response to loss and reinfusion of 25 % of blood volume before and after cutting of the superior laryngeal nerve bilaterally and after bilateral vagotomy in a S D rat

The effect of sectioning of vagus nerves

After sectioning of the cervical vagus bilaterally the S D rats exhibited unchanged or raised heart rate on hemorrhage (Fig 4 and 6 and Table I

The S G S rats usually exhibited a less pronounced acceleration of the heart rate on hemorrhage after bilateral vagotomy The reduction corresponds largely to the acceleration appearing in connection with vagotomy

The effect of atropine

If 0 25—0 5 mg atropine is injected i p into S D rats prior to the bleeding, the lowering of heart rate is almost eliminated (Fig 5 and 6)

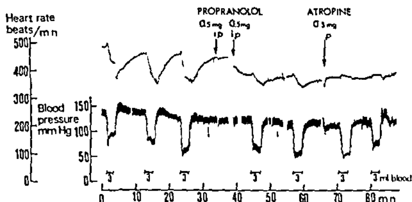


Fig 5 Effect of propranolol and atropine on the heart rate response to loss and reinfusion of 25 % of blood volume in a S D rat

In S G S rats atropine was found to cause some reduction of the rise of pulse rate on hemorrhage which appears to correspond to the acceleration induced by atropine itself

The effect of propranolol

Propranolol in a dose of 1 mg i p reduces the bradycardia on hemorrhage in S D rats (Fig 5)

The rise of heart rate in S G S rats on hemorrhage is mostly inhibited by propranolol If both propanolol and atropine are given, the change of heart rate is eliminated both in S D and S G S rats

The effect of exclusion of impulse transmission via the laryngeal communicans

The afferent vagus nerve fibres from mediastinum heart, lungs trachea and oesophagus run partially via the recurrent and superior laryngeal nerves to the cranial part of the cervical vagus The impulses transmitted via the communicans can be excluded by section of the recurrent nerve or the superior laryngeal nerve

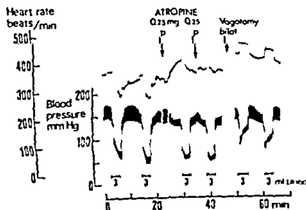


Fig 6 Effect of atropine and bilateral vagotomy on heart rate response to loss and reinfusion of about 25 % of blood volume in a S D rat

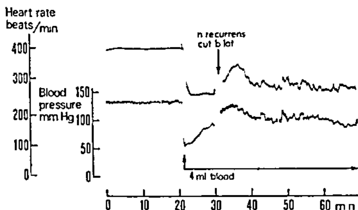


Fig 7 Effect of cutting of the recurrent nerve bilaterally on heart rate and blood pressure in a hypovolaemic S D rat (4 ml of blood withdrawn)

In S D rats this usually eliminates the lowering of heart rate due to haemorrhage (Fig 4 and Table I) Sectioning only on one side eliminates most of the lowering of the heart rate

In S G S rats the exclusion of the laryngeal communicans has no obvious effect The exclusion of the laryngeal communicans under ordinary conditions usually causes a rise of the heart rate with slow onset which is specially distinct in S D rats If exclusion is done after a loss of about 25 % of the blood volume, the heart rate rises rapidly and then falls slowly (Fig 7) At the same time it is noticeable that heart rate and blood pressure show greater variations than before sectioning There is apparently no direct relation between the variations of heart rate and of blood pressure

Comments

The reported observations show that in the rat a reduction of blood volume with consequent drop in blood pressure may cause either an increase or a decrease of the heart rate Under pentobarbital anesthesia S D rats have practically always exhibited bradycardia after a loss of 20—36 % of the blood volume whereas rats of Swedish germ free strain have equally constantly exhibited tachycardia Sectioning of the vagus and administration of atropine and propranolol have shown that the lowering of heart rate is mediated chiefly via vagal efferents but partly also by in hibit on of the sympathetic tone of the heart The rise of heart rate seems to be caused by the reverse conditions It is thus inhibited to a predominant extent by propranolol but is completely blocked after administration also of atropine

We have interpreted the effect of anesthesia and of total or partial exclusion of the baroreceptors in the carotid sinus as being d e to a change of the excitatory state of higher centres On increase of the sympathetic tone as during ether nar cosis or on inhibition of the vagal tone by ligat on of the carotid arteries the vagal depression of the heart on hemorrhage would thus be wholly or partially inhibited

Exclusion of vagal afferents via the laryngeal communicans may entirely inhibit bradycardia on hemorrhage From this we have drawn the conclusion that the afferent link in the vagal reflex induced by hemorrhage runs via the communicans In a subsequent paper (Castenfors, Knutsson and Sjostrand 1972) it will be shown that changes of impulse discharges may be recorded in the recurrent or superior laryngeal nerve on variations of the blood volume

As an explanation of the reported observations the hypothesis is advanced that, on variations of the blood volume, two antagonistic mechanims are activated—one increasing the sympathetic tone and inhibiting the vagal tone on the heart, the other increasing mainly the vagal tone The former corresponds to the effect of baro- and stretch receptors on the high pressure side, the latter seems not previously to have been described It is presumed to be elicited from the low pressure side According to the extent of the hemorrhage and the excitatory state of the higher nervous centres, one or the other mechanism will predominate

The observation that the heart rate is increased on exclusion of the afferents in the laryngeal communicans indicates that normally a tonic influence is exercised by the postulated receptors After hemorrhage this effect is accentuated The observation that the exclusion is followed by more pronounced spontaneous variations of heart rate and blood pressure than before indicates that the postulated regulatory mechanism on the low pressure side contributes to the blood pressure homeostasis

For discussion of the results see the subsequent paper

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Circulatory Control via Vagal Afferents

II. Effects of hemorrhage on afferent discharges via the laryngeal communicans in the rat

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Abstract

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Recordings were made of afferent impulses passing through the communicating branch between the superior and inferior laryngeal nerves and in the recurrent nerve after section of the vagus Two types of discharges varying with changes of the blood volume were recorded

1 Continuous nerve impulses which are increased in frequency on hemorrhage

2 Repetitive burst of spikes with a heart rhythm which starts at the first heart sound

... receptors of type B Their function is postulated to be opposite to that of the former receptors thus increasing the heart rate with increasing central blood volume

In the previous paper observations were reported on changes of the heart rate induced by hemorrhage in the cat (Castenfors and Sjostrand 1972) In Sprague Dawley rats, anaesthetized with pentobarbital, the usual response is a lowering of the heart rate This can be eliminated by, *inter alia* bilateral sectioning of the recurrent nerve or the superior laryngeal nerve, thus excluding afferent impulses via the communicating branch between the superior and inferior laryngeal nerves It is assumed that this effect is due to the exclusion of vagal afferents from receptors on the low pressure side of the vascular system which exercise an antagonistic effect on the baroreceptor mechanism on the high pressure side

The present paper reports a study of the afferent impulses transmitted via the laryngeal communicans and the effect of haemorrhage on these It was demonstrated that in some groups of nerve fibres the discharges vary with variations of the blood volume

Material and Methods

Adult rats (200—350 g weight) predominantly of the Sprague-Dawley strain but some of the Swedish germfree strain S G S (Gustafsson 1948), were used Anesthesia was induced with sodium pentobarbitone given i p 5—8 mg/100 g b wt, and maintained with the same drug given as necessary Two catheters were inserted into one carotid artery and one femoral artery or into both femoral arteries One of these was connected to a differential transformer transducer (Elema, EMT 490 A) to monitor the arterial blood pressure for control purposes withdrawl of blood (2—8 ml) was done through the other ECG were recorded with needle electrodes and the signals were amplified in a differential amplifier (Grass P 9) and monitored on one of the beams of a double beam oscilloscope

Heart sounds at nominal frequencies 50, 100 200 and 400 c/s were recorded with a conventional phonocardiograph (Elema Mingograph 42+42 B) with the microphone fixed to the skin in the precordial area

The superior laryngeal nerve on one side was exposed through a mid line incision over the larynx and retraction of skin and muscles After being freed from the carotid sheath the nerve was dissected over a length of more than 1 cm and cut at its entrance into the vagus nerve The communicating branch between the superior and inferior laryngeal nerves was laid bare by removing the cricothyroid muscle Under a binocular microscope (×10) all branches to the larynx, both those from the superior laryngeal as well as from the inferior laryngeal nerve were carefully cut avoiding stretch or injury to the thin communicating branch The superior laryngeal nerve was then led over a pair of silver input electrodes to record the afferent impulses passing through the communicating branch In a few experiments recordings were made from the recurrent laryngeal nerve or filaments of this nerve after section of the vagi

The input electrodes led to a conventional differential amplifier The signals were monitored on a double beam oscilloscope and simultaneously fed to a loudspeaker and to a FM tape recorder (Tandberg TIR 100) for later display and analysis

Usually there was a slightly irregular tonic discharge of spikes Often these discharges showed a respiratory rhythm in some there was a cardiac rhythm Occasionally all these types of nerve activity could be obtained from one and the same nerve by altering the position of the nerve on the recording electrodes or by splitting of the nerves into thinner filaments Hence the differences in discharge pattern were the result of random contact with various types of afferent fibres Consequently it was not possible to recognize any differences in discharge pattern either between the two strains of rat used or in the recordings from the superior and the inferior laryngeal nerves Hence no distinction has been made between the results obtained in different strains or at a different recording sites

To render easier the visualization of alterations in mass activity the signals were fed to a resistor-capacitor integrator (Grass 7 P 3 B with a time constant of 0 2 sec providing a unidirectional signal approximately proportional to the average amplitude of the signals In all successful recordings were obtained in 25 animals

Results

The recordings in the superior laryngeal nerve of afferent impulses transmitted via the laryngeal communicans or in the recurrent laryngeal nerve reveal a continuous flow of impulses in several nerve fibres Besides a maintained slightly irregular tonic discharge (Fig 1 A) most recordings show a respiratory rhythm with enhanced afferent inflow during inspirations In several specimens bursts of impulses with a cardiac rhythm were observed (Fig 1 and 2 A The present paper is restricted to a presentation of the changes of the tonic spike discharges in response to hemorrhage and to data on afferent nerve impulses activated synchronously with the heart rhythm

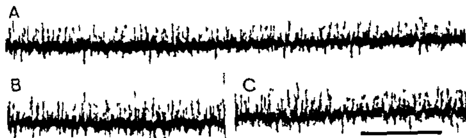


Fig 1 Tonic impulse discharges in filament of the left superior laryngeal nerve in rat during hemorrhage Communicating branch between superior and inferior laryngeal nerve intact all branches to larynx cut A control B and C after withdrawal of 2 ml and 4 ml of blood respectively via femoral artery Time bar in C, 200 ms

Tonic discharges

In about half of the recordings with continuous tonic impulse discharges an increase in total impulse activity was observed during and after hemorrhage The typical changes in impulse discharge during withdrawal of blood via one of the femoral arteries are shown in Fig 1 Before withdrawal of blood (A) there was an irregular tonic discharge of spike potentials of several different amplitudes No respiratory or cardiac rhythm could be found in these discharges which apparently arise in several fibres After withdrawal of 2 ml (B) and 4 ml (C) there was a marked increase in the frequency of the discharges both of those with the highest amplitude and of those with lower amplitudes The activity after hemorrhage in this case also showed a respiratory rhythm due to slightly higher impulse frequency during inspirations

In Fig 2 is shown a continuous recording of the integrated neurogram (A) of the afferent activity transmitted via the communicans during withdrawal and reinfusion of blood in another experiment As can be seen by comparing the average nerve activity to volume of blood withdrawn (B) there was a signifiant increase after withdrawal of less than 2 ml and a further somewhat irregular increase during the withdrawal of up to 3 ml There was no further increase in activity during the withdrawal of an additional 2 ml of blood thus decreasing the volume of blood by altogether 5 ml During the first period of reinfusion there was a further in

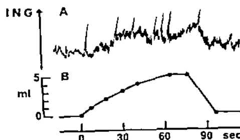


Fig 2 A Integrated neurogram ING from the left superior laryngeal nerve during withdrawal and reinfusion of blood Upward deflection indicates increased average spike activity Large peaked upward deflections correspond in time to exaggerated inspiratory movements B Volume of blood withdrawn from the circulatory system

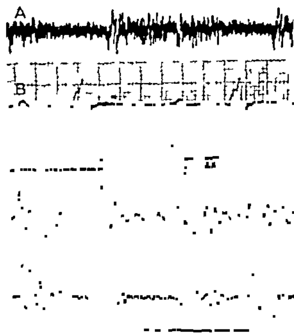


Fig. 3. Recordings of impulses with a cardiac rhythm (A), ECG (B) and heart sounds at frequencies 200 c/s (C) and 400 c/s (D). Time bar in D 100 ms.

crease in activity and then towards the end of reinfusion the activity successively diminished reaching approximately the same level of average activity as before the blood loss.

In most of the other recordings with an altered tonic discharge during hemorrhage a significant increase in average activity, as revealed by integrated neurograms, was obtained on withdrawal of about 2 ml of blood. Usually an additional withdrawal of blood up to a total blood loss of abut 4—5 ml further increased the average activity.

The recording of mass activity from a nerve with several active fibres as used in the present study does not allow [illegible] distinction between spikes arising in individual fibres. Hence it is not possible to decide with full certainty whether or not the rise in total activity is [illegible] frequency in fibres active at normal blood volume or [illegible] previously inactive fibres. The fact that spike amplitudes after withdrawal of blood [illegible] not significantly altered as compared to those observed [illegible] periods favours the view that the response to hemorrhage is mainly [illegible] frequency of discharge in fibres active at a normal blood volume. During [illegible] of blood spikes of amplitudes not seen either during control periods or during withdrawal of blood have been observed in several experiments. Hence it may [illegible] assumed that a group of receptors not active at normal blood volume or during [illegible] loss may be activated during reinfusion of blood.

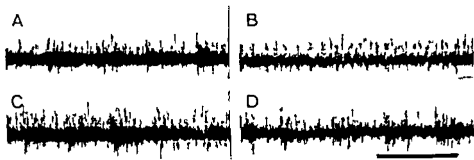


Fig 4 Changes in impulse firing during hemorrhage in preparation with both continuous impulse discharges and a cardiac rhythm Before (A) after withdrawal of 6 ml of blood (B) during the end (C) and immediately after (D) the reinfusion of blood Time bar 200 ms

Discharges with a cardiac rhythm

Firing of bursts of impulses regularly appearing in the same phase of the cardiac cycle was observed in 5 animals A typical discharge recorded simultaneously with ECG is shown in Fig 3

The figure also includes recordings of the heart sounds obtained from another animal with identical heart rate From these recordings it becomes apparent that the burst of impulses starts at the middle of the first heart sound and continues through the second and fourth sounds ending a short time after the latter If the impulses originate in the heart or its immediate vicinity they are delayed by the time of conduction to the site of recording in the superior laryngeal nerve Hence they can be expected to start and end earlier at the site of origin than in the recording Though the conduction velocity of the fibres is not known it seems reasonable to expect a conduction time of 5—20 ms from data on conduction times in type B fibres (Paintal 1953) Consequently the impulses may be assumed to start at the isometric contraction of the ventricles and end approximately at the fourth heart sound The period during which the fibres are activated thus coincides with the period of inflow of blood to the atria and ventricles from the great veins (*c f* Kalmanson Veyrot and Chiche 1971)

During withdrawal of blood the activity synchronous with the heart rhythm invariably disappeared In some recordings the tonic impulse discharge was enhanced concomitantly with the disappearance of a cardiac rhythm This is illustrated by Fig 4 showing bursts of spike discharges with a cardiac rhythm at normal blood volume (A) and a tonic high frequency discharge without any cardiac rhythm recorded after withdrawal of 6 ml of blood (B) During reinfusion (C) a cardiac rhythm is reattained against a background of tonic activity whereas after the end of reinfusion (D) the impulse discharges attain the same pattern as during the control period

In Fig 5 are shown recordings during withdrawal of blood in a specimen without any tonic discharges and consequently the changes in activity synchronous with the

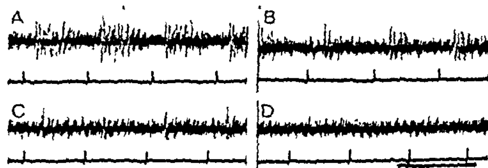


Fig 5 Response to hemorrhage in whole nerve with impulse discharge with a cardiac rhythm A control before hemorrhage Withdrawal of blood was 4 ml in B, 4 5 ml in C and 5 ml in D Time bar 200 ms

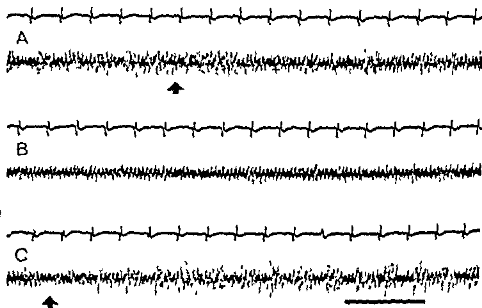


Fig 6 Recordings in preparation with impulse discharges with a cardiac rhythm during insufflation of air via the tracheal cannula Start of insufflation at arrow in A during insufflation in B opening of air passage at arrow in C Time bar 400 ms

heart beat during withdrawal of blood are easier to discern As is indicated by these recordings there was a successive reduction of the number of impulses in each burst as the blood volume was reduced by about 4 -4 5 ml (B C) and concomitantly the impulses appeared at a greater distance after the QRS complex After withdrawal of 5 ml (D) the activity was completely abolished but reappeared after reinfusion

In 4 expts in one animal the activity synchronous with the heart rhythm was also abolished by increased intrapulmonary pressure This is illustrated in Fig 6 show-

ing a continuous recording of ECG and impulse activity in the superior laryngeal nerve before, during and after insufflation of air into the tracheal cannula As seen in the figure the discharges successively diminished and finally disappeared unmasking impulses of low amplitude with a respiratory rhythm After the insufflation the cardiac rhythm was restored

Discussion

Recording of neurograms from the laryngeal communicans appears to have been done earlier by Andrew (1954), Mc Cubbin *et al* (1958) and Krieger and Marseillan (1963) Andrew recorded in rats activity synchronous with the heart beat which was limited to the first part of ventricular systole but sometimes continued to the succeeding P wave The discharge was assumed to derive from baroreceptors in the aortic wall and to have a depressor effect on the heart. By means of electrical stimulation he showed it to be probable that the communicans also carries afferent fibres from the oesophagus and trachea Mc Cubbin *et al* (1958) and Krieger and Marseillan (1963) have adopted Andrew's opinion about the existence of aortic depressor fibres in the laryngeal communicans

Anatomical investigations in the dog also appear to show that in the communicans there run fibres from the oesophagus and trachea (Lemere 1932) The functional significance of the communicans, however, has not been analysed, though it is stated that sectioning of the communicans is necessary in order to induce neurogenic hyper tension in the rat (Krieger 1964)

The activity synchronous with the heart rhythm reported in the present paper is not in accord with the activity in the records published by Andrew (1954) and seems on the whole not to be related to changes of the systolic pressure It seems to cor respond to the activity first described by Whitteridge (1947) and ascribed by Paintal (1953) to atrial receptors of type B They are considered to correspond to the receptors demonstrated by Nonidez (1937) and later by Coleridge *et al* (1957), partly located subendothelially at the junction of the large veins with the atria on the right and left side and partly in the myocardium between the muscular fibres The recep tors are assumed to be stimulated by stretching of the atrial wall on inflow of blood Reduction of the activity from atrial receptors of type B on hemorrhage has been observed earlier (Henry and Pearce 1956 Gupta *et al* 1966) and has been related to diminished filling of the atria

It seems obvious from earlier investigations that there are atrial receptors of type B which are stimulated by stretching of the atrial wall and which appear coincidently with the v wave in the atrial pressure curve However the activity in our records is more consistent with the time for the outflow of blood from the larger veins to the heart For the pressure curve, recorded directly and without damping Wiggers 1934) shows a rapid decline after the isometric relaxation phase of the ventricles *i e* during their rapid filling phase and thereafter the atrial pressure rises only slowly until atrial systole begins The decline of atrial filling after the isometric relaxation

phase of the ventricle would be reflected in the neurogram if the discharge was related to stretching of the atrial myocardium. The very short diastole in our records does not enable us to take a definite standpoint at present, but we would establish that the receptors firing discharges synchronous with the heart rhythm via the laryngeal communicans are activated in one way or another by the blood flow from the larger veins to the heart. This starts immediately after the isometric contraction phase of the ventricles and terminates with atrial systole (Kalmanson *et al.* 1971) *i.e.* at the appearance of the fourth heart sound. The finding that with a large blood volume, the activity starts earlier during ventricular systole, as has been observed previously (Kramer 1959), indicates that at high pressure on the filling side the inflow of blood takes place during the relaxation of the atria. Cessation of the activity at a given loss of blood volume, as also the successive shortening of its duration during hemorrhage, indicate that the inflow of blood to the heart must be of a given velocity for the receptors to be stimulated.

The activity synchronous with the heart rhythm is inhibited by insufflation of air into the lungs as observed earlier. This is explained by the fact that the intrathoracic rise of pressure inhibits the inflow of blood into the thorax and thus reduces the central blood volume.

The continuous nervous impulses which are increased on a reduction of the blood volume do not appear to have been described earlier. They appear simultaneously with but also in the absence of the discharges synchronous with the heart rhythm. On simultaneous recording of these two activities it is found that the continuous discharge increases at the same time as the activity synchronous with the heart rhythm diminishes during hemorrhage.

The location of the allied receptors on the low pressure side is suggested by the following. (1) Their discharges are transmitted through the laryngeal pathways known to carry afferents from trachea, bronchi and the lungs. (2) The discharges do not show changes with the heart rhythm. (3) The receptors respond to a small decrease of the blood volume which is compensated primarily by a decrease of the volume on the low pressure side. (4) Their functional effect in causing bradycardia (see below) is opposite to that of the baroreceptors on the high pressure side. It appears most probable therefore that both activities described here are elicited by receptors on the low pressure side, one group being activated synchronously with the inflow of blood to the heart preponderatingly at large central blood volume and the other group the continuously firing receptors at decreased central blood volume. At this stage of knowledge we would suggest designating the first group high level receptors (HL) and the second low level receptors (LL) of the low pressure side.

The functional significance of atrial receptors of type B is generally considered obscure. It has been thought that like atrial receptors of type A and the receptors localized in the ventricular walls they cause reflex bradycardia, systemic hypotension and venodilatation *i.e.* relieve the load on the central vessels and heart (Folkow *et al.* 1965). Nonidez (1932) assumed that the receptors described by him at the junction of the veins in the two atria constituted the basis for the Bainbridge reflex

Paintal (1953) and Coleridge *et al* (1957), however, point to the uncertainty concerning the existence of the Bainbridge reflex Henry and Pearce (1956) and Gupta *et al* (1966) presume that cardiac stretch receptors induce changes in urine excretion and thereby have a regulative significance for the blood volume

From the observations reported here it is apparent that the HL-receptors are active at ordinary central blood volume Their function can therefore not be limited to protection of the heart against overloading Whether the specific stimulation is stretching of the atrial walls or emptying of the supplying veins, the activity appears to correspond to the speed and time for filling of the heart *i e* the stroke volume Within certain limits, therefore, this may be expected to be related to the number of impulses during the cardiac cycle A slowing of the heart rate would increase the blood volume on the filling side and also the time for diastole Consequently if the receptors influence the vegetative tone of the heart in a parasympathetic direction they would provoke a vicious circle It is therefore assumed that these receptors influence the heart activity in a sympathetic direction. The existence of receptors with a sympathetic effect and located at the junction between the pulmonary veins and the left atrium is also well established (Ledsome and Linden 1964 Carswell *et al* 1970 and Edis *et al* 1971)

The LL-receptors signal a lowering of the level of the central blood volume and well correspond to the basis sought for the increased vagal tone on hemorrhage under some conditions (Castenfors and Sjostrand 1972) The impulse frequency thus increase with reduction of the blood volume After the end of blood letting the increased activity remains unchanged

On this assumption the two groups of receptors would constitute the afferent basis for the postulated antagonism on the low pressure side to the baroreceptors on the high pressure side (Castenfors and Sjostrand 1972) The purpose of the postulated mechanism must consequently be viewed in relation to the baroreceptor mechanisms on the high pressure side

An increase of the blod volume on the low pressure side may be expected through the action of the HL-receptors to cause an increase of heart rate and cardiac out put which at unchanged resistance on the arterial side leads to an increase of pressure and activation of baroreceptors in the aortic wall and carotid sinus This would increase the vagal influence on the heart and reduce the resistance in the systemic circulation A compromise occurs at a somewhat elevated arterial pressure somewhat increased cardiac output and at a given redistribution of the blood between the central part of the circulation and the periphery If the resistance in the systemic circulation is reduced simultaneously with the increase of the central blood volume *e g* on muscular activity the arterial buffer mechanism is not or only to a small extent brought into play and consequently the increase of cardiac output is retained

Hemorrhage may be expected to result in increased vagal tone via LL receptors and thus to provoke a reduction of heart rate and cardiac output This leads to a lowering of the arterial pressure and reduced activity from baroreceptors on the

high pressure side so opposing the rise of vagal tone and increasing the resistance in the systemic circulation. A compromise is attained at a somewhat lower arterial pressure, reduced cardiac output, and on a redistribution of blood between the central and peripheral parts of the vascular system. The heart rate may increase, *decrease or remain unchanged depending on the loss of blood and the excitatory state* of the cardio-vaso-motor centres. The antagonistic regulation may be expected to give to the circulation the stabilization which appears to be disturbed on sectioning of the vagal afferent fibres via the laryngeal communicans (Castenfors and Sjöstrand 1972), as is also the case on exclusion of the baroreceptors on the arterial side (Thomas 1944). This outline of the adaptation of the circulation also provides an explanation of the contradictory statements concerning the Bainbridge reflex and of its dependence on the heart rate (Coleridge and Linden 1955, Jones 1962). The so called vaso-vagal reflex and the eliciting of syncope on orthostatic stress and in patients with dumping syndrome (for references see Castenfors and Sjöstrand 1972) also find an explanation.

Hitherto it has been difficult to relate the vagal afferents to the regulation of the circulation. This is because it has been impossible to separate vagal afferents and efferents in experiments with nerve section and stimulation with the exception of the peripheral nerve branches and when the depressor fibres from the heart and aorta have constituted an isolated nerve, the depressor nerve. The recurrent nerve and the superior laryngeal nerve with afferents via the laryngeal communicans, however, appear to offer hitherto unutilized means of studying the significance of some groups of vagal afferents for the regulation of circulation and respiration.

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The Role of Cl⁻ and other Anions in Active Na⁺ Transport in Isolated Frog Skin

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Abstract

HUF E G *The role of Cl and other anions in active Na⁺ transport in isolated frog skin* Acta physiol scand 1972 84 366—381

Skins of several amphibian species were kept between hemi chambers in solutions of identical SO_4^{2-} Ringer s solutions with or without mannitol (SM S) When steady state was reached the SO_4^{2-} ions were replaced by equivalent amounts of one of the following anions (A) Cl, HCO_3 NO_3 Replacements were made in sequence on the outside (o) the inside i and both sides of the skin with periods of SO_4^{2-} solutions on both sides between the several phases. In other experiments skin pairs were set up for simultaneous Na [illegible] and Cl^{35} influx and out flux measurements In all experiments measurements on short circuit current (SCC and open skin PD (E) were made and from this total skin conductance (G_T) data were calculated as were values for E_{Na} and G_{Na} From these data it is concluded that Cl (with SM_o leads to stimulation of Na influx and net Na flux resulting from epithelial swelling increase in SCC, E G_T E_{Na} G_{Na} The order of effectiveness of A_i on electrical parameters was Cl > NO_3 > HCO_3 Cl_o with Cl) resulted in a) "shunting of E_{Na} b) depression of Na influx of SCC E E_{Na} G_{Na} c Cl with SM_i) seemed to stimulate Na influx but the flux data were as yet inconclusive There were marked skin species differences Best recoveries of starting values were obtained with skins of *R esculenta* Increases or decreases in Na influx did not correlate with changes in Na outflux Cl influx and Cl outflux Possible sites of A membrane interactions involving the Na transport system are discussed Work by others is confirmed that in skins of *R esculenta* bathed in SO_4^{2-} Ringers with 4 mM Cl Cl is actively transported in the inward direction at a level of approximately 10% of the active inward Na transport

In a previous study (Smith Hughes and Huf 1971 we have compared the permeability of frog skin (*R pipiens* for several anions Cl SO_4^{2-} HCO_3 and NO_3 It was concluded that Cl readily permeates the skin (as was expected and that the other mentioned anions diffuse only very slowly across the skin In these experiments the skins were exposed to 135 mosm sulfate Ringer s on one side and on the opposite side, to 195 mosm Ringer s solution made with NaCl or $NaHCO_3$ When chloride Ringer's was used it was observed that upon replacing sulfate Ringer s on the outside by chloride Ringer s (keeping sulfate Ringer s on the inside the skin PD precipitously decreased In most cases the outside turned electrically positive with

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respect to the inside. The reversed skin PD's were often maintained for several hours. On the other hand, if the inside sulfate Ringer's was replaced by chloride Ringer's (keeping sulfate Ringer's on the outside) very little change in PD was seen during a 15 min period. In some cases an insignificant rise in the skin PD was noticed. By applying the electrical skin model proposed by Ussing and Zerahn (1951) and supposing that in the "open skin" Cl⁻ ions act as a "shunt" to the potential generated by the Na^+ transporting system it was expected that, with chloride Ringer's on the inside, the PD would significantly rise, as it decreases with chloride Ringer's on the outside. This inconsistency suggested that Cl⁻ ions besides their shunting effects must have other effects on the skin which, of course, are not ruled out by the concept of Ussing and Zerahn referred to above. There have appeared in the literature indeed already several papers which deal with these other effects of anions in electrogenesis in frog skin and also urinary bladders (see Discussion). Notable among these publications is the study by Ferreira (1968) who found that skins in chloride Ringer's on both sides give a significantly greater short circuit current than can be drawn from skins in sulfate Ringer's, provided that the latter is made isomotic to chloride Ringer's by adding, *e.g.*, mannitol.

The present paper presents a systematic study of the separate electrochemical effects of Cl⁻ ions on the two sides of isolated skins of several species of amphibia. For comparisons, the effects of HCO_3^- and NO_3^- ions were also investigated.

Methods

Animals. Frogs of the species *R. esculenta*, *R. temporaria*, *R. pipiens*, *R. catesbiana* and toads (*Bufo bufo*) were stored in shallow bins with water in a cold room (4° C). Before use the animals were adapted for 1 to 2 days to room temperature. The animals were sacrified by decapitation and double pithing. All Na^{24} and Cl^{36} flux experiments were carried out on skins of *R. esculenta*. The experiments were conducted during all seasons in 1969 and 1970.

Electrical Measurements. The method used was essentially that described by Ussing and Zerahn (1951). The following modifications were applied: 1) In solutions containing no added NaCl, the short circuit current was applied via agar bridges matching in salt composition with the solutions applied to skins. Abdominal skins (3.8 cm²) exposed to NaCl containing solutions were shortcircuited via Ag/AgCl electrodes placed directly into the hemi chambers (17.2 ml). Calculation showed that even at the lowest [Cl⁻] = 4 meq/l used the [Ag⁺] in the fluid compartments could not have exceeded 3×10^{-5} M with negligible rise in [Ag⁺] in the anodic compartment during a one hour period of short circuiting. At this [Ag⁺] level no damaging effect on skins were noticed as tested in separate experiments. Cl⁻ flux measurements were not interfered with, as judged by the fact that in all salt solutions with low or high [Cl⁻] the sum of the Na⁺ and Cl⁻ fluxes equalled the short circuit current equivalent (SCCE). 2) In all experiments skin potentials were measured by replacing agar bridges by small funnels with near capillary tips turned towards the surfaces of the skin nearly touching them. The funnels contained the same solutions used on the respective side of the skin. Small cotton plugs were inserted into the stems of the funnels. Pencil type commercial saturated KCl calomel half electrodes were used to lead off the PD to a high impedance recording recorder. No KCl leakage into the solutions in the skin chambers was detectable in a 4 hour period. By this technique the occurrence of diffusion potentials was minimized.

The skins were kept shorted (with manual and/or continuous automatic control) throughout the experiment. At intervals the SCC was briefly interrupted for open skin PD measurements. Occasionally PD transients were seen. In these cases the SCC was interrupted for exactly 1 min and an average PD was calculated. The skin edge/surface area ratio (E/S) was calculated as 1.8 cm⁻¹ and chamber edge damage to ≅ 10%. According to Dobson and Kidder (1968) this had a negligible effect only on PD's and hence on skin conductances (short circuit current/PD).

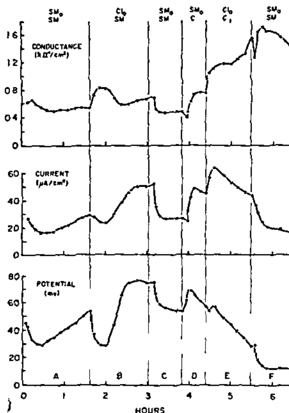


Fig 2 Effects on open skin potential short circuit current and total skin conductance of sequential replacement of sulfate mannitol solution (198 mosm) by chloride solution (196 mosm) on the outer and inner surface of the abdominal skin of *Rana temporaria* Single experiment

(phases A—B) there occurred a considerable increase in SCC and a rise in PD after a transient decrease Later by replacing SM_o SM by SM Cl (phase D) the SCC rose as did the PD to a small degree Then by changing from SM_o Cl to Cl Cl there was a precipitous drop in both SCC and PD conductance remaining nearly the same It is interesting to note that later when SM SM was once again changed to Cl SM_i (phases F—G both SCC and PD drastically decreased and changed sign By comparing the identical phases B and G it is seen that during a period of 3 hours the characteristics of this skin have greatly changed The rise in PD and SCC (seen in phase B) did not recur in phase G Such observations with signs reversing occur more frequently in skins of *R pipiens* (Smith Hughes and Huf 1971) than with skins of other frog species but reversal in sign of PD and SCC were occasionally also seen in skins of *R esculenta* when the starting values were low

2 *Electrical responses of skins when using mannitol (M) free SO_4^{2-} solutions*

Omitting M from the outside sulfate solution had no effect on PD SCC and conductance (6 experiments *R esculenta*) Omitting M from the inside solution greatly increased the values for these electrical parameters (MacRobbie and Ussing 1961) Changing the solutions from S S (mannitol free to S Cl further but only slight

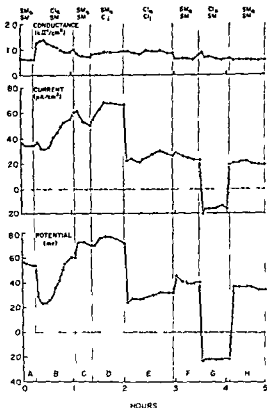


Fig 3 Effects on open skin potential, short circuit current and total skin conductance of sequential replacement of

ly increased the values for the electrical parameters In this situation, $[Na^+]_o = [Na^+]_i$, but the outside solution was 134 mosm, the inside solution 196 mosm Four further experiments (*R esculenta*) were carried out with hypotonic, 67 mM NaCl solution (134 mosm) A typical result of such an experiment is shown in Fig 4 Attention is called to the following a) S greatly increased PD, SCC, conductance (phases B—C) b) There was only a moderate additional increase in the electrical parameters when S_i was replaced by 67 Cl_i (phases C—D) as was the case with 104 Cl_i, mentioned above In the experiment of Fig 4, $[Na]_o$ was somewhat greater than [Na] c) With 67 Cl_i on both sides PD, SCC, conductance changed but little Usually there was a transient decrease followed by an increase d) Recovery of original values for the electrical parameters was good

3 *Electrical response of skins following replacement of $SO_4^=$ by NO_3 or HCO_3*

To test the specificity of actions of Cl on the electrical responses of skins described under (1), 11 expts with Cl-free NO_3 solutions, and eleven with Cl free HCO_3 solutions were carried out on skins of *R esculenta* The sequence of solution changes was the same as the one shown in Fig 1 to 3 The results were qualitatively very similar to those shown in Fig 1—3 There were quantitative differences, how-

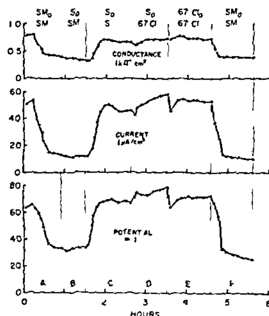


Fig. 4. Effects on open skin potential, short circuit current and total skin conductance of sequential replacement of sulfate solution with and without mannitol (198 and 134 mosm respectively) by chloride solution (134 mosm) on the outer and inner surface of the skin of *Rana esculenta*. Single experiment.

comparing the effects of HCO_3, NO_3, Cl when acting on the inside of the skin. In all cases when changing solutions from SM, SM (Cl free) to SM, A_i (A = anion) the rise in SCC was in the proportion of 1 : 1.5 : 4 for HCO_3, NO_3, Cl (9 Cl experiments) respectively. Elevation in skin PD was greatest with Cl (+72 %) and about +10 % in the cases of NO_3 and HCO_3. Skin conductance increased by approximately 30 % when Cl or NO_3 was used and by 10 % when HCO_3 was used.

In all bicarbonate experiment there occurred a transient increase in potential, current and conductance when changing the solutions from B, B to SM_o, SM. This was not or only to a small degree observed in the chloride and nitrate experiments. 3 typical examples are shown in Fig. 5.

4. *Results of Na^{24} and Cl^{36} flux measurements*

The results described above suggested that anions, especially Cl, besides their shunting effect may both stimulate and inhibit Na transport. Evidence for stimulatory effects has already been presented by Ferreira (1968). The difference between her studies and the present ones is that by testing the responses to Cl on the inside and the outside of the skin separately it became evident that both stimulatory and inhibitory effects can be elicited at the two sides of the skin.

Because of practical considerations flux measurements had to be limited to studies of two sequential solution changes as given in the top row of Table II and III. E.g. a control phase SM, SM was followed by an experimental phase SM_o, Cl. In paired skins Na^{24} and Cl^{36} were placed on the outside or the inside of the skin to

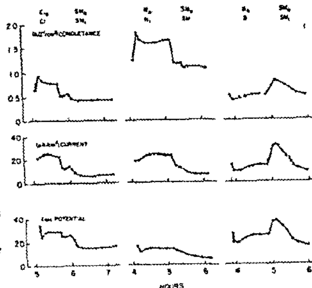


Fig 5 Effects on open skin potential short-circuit current and total skin conductance on three pieces of abdominal skin from three frogs (Rana esculenta) when the solution changes as indicated in the graphs were made All skins underwent a full sequence of solution changes as seen e g, in Fig 1 The data shown represent the terminal phases

obtain simultaneous Na and Cl influxes and outfluxes respectively As is well known the difficulties that arise in paired studies is that even skin pairs which match electrically reasonably well at the start often do not compare well at all as time goes on Therefore many experiments had to be discarded i e those which did not match throughout the experiment to ±10 % This difficulty was especially disturbing in pairs when the solutions were changed from SM, SM, to Cl SM Therefore only two successful experiments of this kind are listed in Table II and III column 3 The electrical data of a total of 17 expts are given in Table II and the flux data obtained on the same skins are listed in Table III

a *Current — flux equivalence* In all cases the sum of the Na and Cl fluxes checked quite well with the short circuit current equivalent (SCCE) This shows that other ions especially $SO_4^{=}$ for which often a considerable concentration gradient across the skin existed did not measureably contribute to the current From the listing of the individual Na and Cl influxes and outfluxes (Table III and the sum of the fluxes given in Table II the percentage contribution of the separate fluxes to the total current was calculated The data are given in the last 2 rows of Table II

b *Stimulation of Na influx with Cl* When changing solution as stated in columns 1 and 2 Table III Na influx and net Na flux significantly increased as did the SCC (Table II) There was no change in Na outflux and Cl influx Cl outflux was greater in the direction of the falling Cl gradient In spite of the favorable condition for Na entry into the skin in the situation (column 2 S 67 Cl (Na — 104 [Na] = 71) the data show that the increment in Na influx and net Na influx was less than in experiments changing solutions from SM SM to SM Cl column 1 $[Na]_o$ = [Na] = 104 mM) Percentage figures for increments in Na influx and

TABLE II Electrical data on paired skins (Na$^+$ and Cl$^-$ fluxes see Table III) The current measured … alent cal … currents … ta given in

	1	2	3		4
Experiment (Control)	SM$_o$ Cl$_i$ (SM$_o$ SM$_i$) 198 mosm	S$_o$ 67 Cl$_i$ (S$_o$ S$_i$) 134 mosm	Cl$_o$ SM$_i$ (SM$_o$ SM$_i$) 198 mosm		Cl$_o$ Cl$_i$ (SM$_o$ Cl$_i$) 198 mosm
Number of pairs	6	5	1	1	4
μA/cm^2	27.6±3.9* (12.3±3.2)	43.0±3.4† (30.2±3.5)	5.6 (8.0)	38.2 (42.5)	20.4±5.0* (40.2±6.5)
mv	32.8±4.2* (18.8±5.1)	61.5±3.1† (50.7±5.7)	11.3 (21.2)	58.4 (67.6)	37.8±4.8* (50.3±6.0)
kΩ^{-1}/cm^2	0.84±0.13† (0.65±0.08)	0.70±0.06† (0.60±0.06)	0.50 (0.38)	0.65 (0.63)	0.78±0.06 (0.80±0.06)
SCCE	1.03±0.14* (0.46±0.12)	1.60±0.15† (1.13±0.13)	0.21 (0.30)	1.42 (1.58)	1.10±0.19† (1.50±0.25)
Σ of fluxes (Table III)	1.10 0.13* (0.39±0.11)	1.61±0.17* (1.07±0.15)	0.22 (0.30)	1.41 (1.51)	1.16±0.22 (1.64±0.18)
% Na$^+$ current	$\overrightarrow{67}^{1}$ $(\overrightarrow{113})^{1}$	$\overrightarrow{91}^{1}$ $(\overrightarrow{103})^{1}$	$\overrightarrow{232}^{1}$ $(\overrightarrow{123})^{1}$	$\overrightarrow{111}^{1}$ $(\overrightarrow{105})^{1}$	$\overrightarrow{91}^{1}$ $(\overrightarrow{92})^{1}$
% Cl current	$^{o}\overleftarrow{33}$ $(\overrightarrow{13})^{1}$	$^{o}\overleftarrow{9}$ $(\overrightarrow{3})^{1}$	$\overrightarrow{132}^{1}$ $(\overrightarrow{23})^{1}$	$\overrightarrow{11}^{1}$ $(\overrightarrow{5})^{1}$	$^{o}\overleftarrow{9}$ $^{o}(\overleftarrow{8})$

* $P < 0.01$ Data not marked were tested but differences were found statistically
† P 0.02 insignificant (P 0.05

net Na flux of +25 % and +38 % and of +32 % and +68 %, respectively can be calculated This suggests that the degree of stimulation of Na influx and net flux was related to the level of Cl

c Inhibition of Na influx with Cl When changing solutions from SM Cl to Cl$_o$ Cl Na influx was significantly decreased column 4 Table III) as was the SCC (Table II Net Na flux also was depressed Inhibition occurred in all 4 expts *Na$^+$ outflux and Cl in and outflux remained essentially unchanged When chang ing solutions from SM SM to Cl SM* stimulation rather than inhibition of Na influx and net Na flux was seen in 1 of 2 expts with no flux changes in the other (column 3 Table III Many of this type of experiments had to be discarded because of matching difficulties in skin pairs Here the double labelling flux method seems more promising *to obtain conclusive results*

d. *Active inward Cl transport* When the skins were bathed on both sides with 100 mM Cl solution (Cl Cl) Cl influx equalled Cl outflux A small value for net Cl outflux proved to be statistically insignificant When SO$_4$ solution (with or without mannitol) containing 4 mM Cl was placed on both sides of the skin a small

TABLE III Na^+ and Cl^- fluxes in paired skins (electrical data, see Table II) All fluxes are given as $\mu Eq \times cm^{-2} \times h^{-1}$. See also heading of Table II

	1	2	3		4
Experiment (Control)	SM_o Cl_i (SM_o SM_i) 198 mosm	S_o 67 Cl_i (S_o S_i) 134 mosm	Cl_o SM_i (SM_o SM_i) 198 mosm		Cl_o Cl_i (SM_o Cl_i) 198 mosm
Na^+ influx	1.12 ± 0.12* (0.81 ± 0.08)	1.64 ± 0.17† (1.31 ± 0.15)	0.69 (0.53)	1.77 (1.77)	1.30 ± 0.22† (1.71 ± 0.19)
Na^+ outflux	0.38 ± 0.06 (0.37 ± 0.06)	0.18 ± 0.02≠ (0.20 ± 0.02)	0.18 (0.16)	0.21 (0.18)	0.24 ± 0.02 (0.20 ± 0.03)
$\rightarrow i$ Net Na^+ flux	0.74 ± 0.14* (0.44 ± 0.10)	1.46 ± 0.16† (1.11 ± 0.14)	0.51 (0.37)	1.56 (1.59)	1.06 ± 0.24 (1.51 ± 0.20)
Cl^- influx	0.20 ± 0.08 (0.17 ± 0.03)	0.12 ± 0.03 (0.07 ± 0.01)	0.36 (0.15)	0.20 (0.13)	0.27 ± 0.03 (0.22 ± 0.03)
Cl^- outflux	0.56 ± 0.08* (0.12 ± 0.02)	0.27 ± 0.04* (0.03 ± 0.0005)	0.07 (0.08)	0.05 (0.05)	0.37 ± 0.04 (0.35 ± 0.02)
$\rightarrow i$ Net Cl^- flux	(0.05 ± 0.03)	(0.04 ± 0.01)	0.29 (0.07)	0.15 (0.08)	
$o \leftarrow$ Net Cl^- flux	0.36 ± 0.08	0.15 ± 0.05			0.10 ± 0.05 (0.13 ± 0.02)

* $P < 0.01$
≠ $P = 0.03$
† $P = 0.02$
Data not marked were tested but differences were statistically insignificant ($P > 0.05$)

net Cl influx was observed in all 13 expts on short circuited skins (Table III, second row from bottom, "controls") The difference between influx and outflux values was highly significant, $0.005 > p > 0.0005$ In this computation the skins were treated as pairs Compared to the active inward net Na^+ transport, the active inward net Cl transport, on the average was 10 % Active inward Cl transport across skins (*R. esculenta, R pipiens*) in sodium sulfate Ringer's containing 2 mM KCl has earlier been described by Martin and Curran (1966) In their experiments on *R esculenta,* the active Cl transport was about 2.5 % (5 % in skins of *R pipiens* of the active Na^+ transport, calculated from the short circuit current

Discussion

1 *Dependence of Na fluxes on Cl* Replacement of SO_4 by Cl on the inside of the skin invariably led to an increase in Na influx and net inward Na flux Skins in SM_o Cl_i (198 mosm), and skins in S 67 Cl_i (134 mosm showed similar results The existing Na gradient across the skins in S 67 Cl could not have fully accounted for the increased Na influx (see section 4b Results Increase in Na influx was associated with some increase in total skin conductance Rise in SCC when Cl

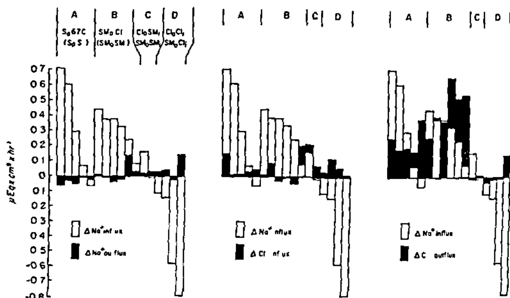


Fig 6 From the data presented in Table III the changes in Na and Cl fluxes obtained in the 17 expts were calculated and plotted A B C D correspond to the column numbers in Table III All bars are to be read from the zero line

is substituted for SO_4 on the inside of the skin has also been seen by Ussing (1965) Voute and Ussing (1968 1970) have observed that the cell layer just beneath the cornified layer appears much denser and shrunk in open and short circuited skins (*R temporaria*) bathed in osmotically uncompensated $SO_4^{=}$ Ringer s instead of Cl Ringer s MacRobbie and Ussing (1961) showed that in *R temporaria* the water permeability of the outer border is only 1/20 of that of the inner border Thus the inside Cl effects seem to be related to swelling of some portion at least of the epithelial cells in the epidermis This conclusion is also reached from the result shown in Fig 4 B C D

Replacement of SM by Cl with Cl) led to a decrease in Na influx and net inward Na flux while total skin conductance remained constant This cannot be explained on the basis of information available at this time Entry of Na into the epidermis appears to be a complex process involving both a saturable and linear rate component (Biber and Curran 1970 Rotunno *et al* 1970) It is possible that Cl changes these rates for Na entry into the epidermis

Experiments to prove conclusively a stimulatory effect of Cl on Na transport failed for methodological reasons However such an effect is suggested by the observations presented in Fig 1 2 and 3 phase B and phase E Fig 1 and 2 The flux data given in column 3 Table III are consistent with this notion

2 *Relations between changes in Na influx to Na outflux and to Cl influx and Cl outflux* Fig 6 shows for all 17 Na and Cl flux experiments that there is no clear relation between changes in Na influx and changes in the other fluxes mentioned

TABLE IV Values for active Na^+ transport potentials (E_{Na}) and Na^+ conductance in the active Na^+ transport pathway (G^a_{Na}) calculated according to Ussing and Zerahn (1951) and using the flux data given in Table III One μeq Na^+/h = 0 0268 mA Last column, total skin conductances (see Table I) calculated as SCC/E, where E is the open skin PD

Solutions	E_{Na} mV	G^a_{Na} $K\Omega^{-1}/cm^2$	SCC/E $K\Omega^{-1}/cm^2$
SM_o SM_i	19 7	0 60	0 65
SM_o Cl_i	27 2	0 73	0 84
S_o S_i	47 4	0 63	0 60
S_o 67 Cl	55 7	0 70	0 70
SM_o SM_i	30 2	0 33	0 38
M_o SM_i	33 8	0 41	0 50
SM_o SM_i	57 5	0 74	0 63
Cl_o SM	53 7	0 78	0 65
SM_o Cl_i	54 0	0 75	0 80
Cl_o Cl_i	42 5	0 67	0 78

This is quite clear from the first and second histogram Likewise upon closer inspection of the third histogram no convincing evidence appears for a correlation between these fluxes Moderate increases in Cl outflux were associated with great increases small increases and decreases in Na influx These results suggest that the pathways for Na influx and those for Na outflux Cl influx and outflux are not identical

3 *Active Na transport potential Na conductance* From the Na flux data (J) given in Table III, values for E_{Na} and G^a_{Na} were calculated (Ussing and Zerahn 1951) The results are shown in Table IV It can be seen that Cl increased or decreased these parameters depending on solutions used and experimental design In other words Cl did alter the active Na transport characteristics Total skin conductance (G_T) was obtained by dividing the open skin PD (E) into the SCC Since in these experiments asymmetrical solution conditions frequently existed one has

$$G_T = \frac{SCC}{E} = \frac{(J_i^{Na} - J_o^{Na}) + (J_o^{Cl} \quad J_i^{Cl})}{E_{Na}G^a_{Na}/(G^a_{Na} + G_j^p) + E_{diff}G_j^p/(G^a_{Na} + G_j^p)} \quad (1)$$

This follows from the network analysis of a modified open skin electrical equivalent circuit (Ussing and Zerahn 1951) adding a diffusion potential (E_{diff}) into the pathway for passively moving ions chiefly Cl Whereas the G_T values Table IV last column), mostly came out somewhat greater than G^a_{Na} there are also some unexpected low values It is assumed that E measured immediately following interruption of the short circuit current is likely to be overestimated because of polarization effects

4 *Interpretation of the electrical responses to anions*

a *Effects of Cl* Fig 1 2 and 3 give evidence for considerable skin species differences in the electrical responses to Cl Recovery of original electrical characteristics was

mostly good in skins of *R esculenta* Skins of *R temporaria* and *R pipiens* underwent permanent changes with time upon sequential changes of solutions as were applied in this study Skins of *R catesbiana* and *Bufo bufo* behaved similarly to skins of *R esculenta* Although flux measurements were limited to only two sequential solution changes, the results clearly showed that Cl_i (with SM_i) stimulated and Cl_o (with Cl_i) inhibited Na^+ flux and SCC There is suggestive evidence that Cl (with SM_i) may stimulate Na flux In addition Cl can also act as a shunt to the Na battery (Ussing and Zerahn 1951) With these multiple actions of Cl on Na^+ transport it is not difficult to realize the complex electrical responses of skins to Cl or Cl_i, such presented in Fig 1, 2 and 3 An exact account of the PD and conductance (G_T) changes observed is impossible by the data obtained There are too many variables (Equation I) which require study by rapid sequential flux and conductance measurements

b *Effects of NO_3, HCO_3* Results similar to those shown in Fig 1 (*R esculenta*) were also obtained with NO_3 and HCO_3 solutions with decreasing effectiveness in the order mentioned Thus the effects of Cl are not specific The equivalent conductances for the three anions are given as 55 8, 50 8 and 30 4 $\Omega^{-1}\times cm^2/eq$ for Cl, NO_3 and HCO_3 respectively This suggests that the decreasing effectiveness is related to the anion permeability of the skin However Smith, Hughes and Huf (1971 have found that skins of *R pipiens* have a very low permeability for NO_3 and HCO_3 in experiments of one hour duration Only when exposed for 12 h to hypotonic solutions was NO_3 seen to permeate the skin (Huf 1952) This suggests but does not prove that permeation of these anions as seems to be the case for Cl (section 2 Discussion) is not a prerequisite for their electrochemical action on skin The anions may interact with the outermost layer of the skin

The results shown in Fig 5 require some comments In contrast to skins in Cl_o Cl or N N skins in B B invariably showed a pronounced transient increase in PD, SCC and G_T when the solution condition SM_o SM was reestablished A satisfactory explanation for this must be postponed until flux data are available This observation may be related to the buffer properties of HCO_3 It is not unlikely that during exposure of the skin to high HCO intracellular P_{CO_2} rose in an attempt to maintain intracellular pH Funder Ussing and Wieth (1967) have shown that HCO_3 can protect skins from the effects of high P_{CO_2} Removal of HCO_3 may then lead to escape of accumulated CO from the cells leading transiently to elevation of intracellular pH Increase in active Na transport at elevated pH of the environment is a well known fact (Ussing 1949 Huf Parrish and Weatherford 1951 Schoffeniels 1956, Snell and McIntyre 1960) It cannot be ruled out that pretreatment of skins with HCO_3 rendered them permeable to SO_4 ions

Similar anion effects as described here were also seen when the serosal side of toad and turtle bladders were exposed to Cl HCO_3 and also to proprionate (Steinmetz Omachi and Grazier 1967 Gonzalez Shamoo and Brodsky 1969 Singer Civan and Sharp 1969, 1970) The role of several organic anions in maintaining skin potential ion transport and metabolism in normal and bromo- or iodoacetate poisoned skins

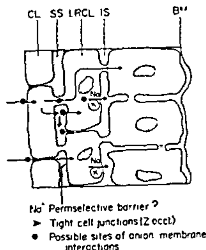


Fig 7 Schematic drawing of the essential features of frog skin epidermis as needed in the context of this study Possible pathways for ion influx into the epidermis and several possible sites for anion membrane interactions are indicated Ion outflux is via one or ... re ... er ... cell ... m ... brane

has been explored many years ago by Huf (1936), Francis and Gatty (1938), Skjelkvåle, Nieder and Huf (1960) Hornby and Thomas (1969) have described decreases in J_{Na}, SCC, E_{Na}, G^a_{Na} and G_T in skins of frogs (*R esculenta, R temporaria*) which were kept for several weeks in 0 7 % saline

5 *Site of anion effects* At present, no conclusive data are available as to the pathways which Na^+ and Cl travel across the skin Both a transcellular pathway (Ussing and Windhager 1964) and an extracellular pathway, along the cell surfaces (Cereijido and Rotunno 1969) has been suggested Thus, the site of the interactions of anions with Na^+ ions remains purely speculative It may be helpful however, to focus at possible sites of interactions based on light and electron microscopic studies on frog skin (Voûte 1963, Farquhar and Palade 1964 Voûte and Ussing 1968) It has been pointed out in the Discussion, section I that the inside Cl effect can be understood on the basis of swelling of the epithelial cells in the presence of this anion Fig 7 summarizes in a schematic way essential features of the electronmicroscopic appearance of the epidermis Only two layers of the stratum spinosum and granulosum are shown The cornified layer is completely separated by the subcorneal space from all other, deeper intercellular spaces The role of this subcorneal space is not clearly understood Skins under influence of aldosterone often have a greatly increased subcorneal space (Nielsen 1969 see also Larsen 1970) Existence of a diffusion barrier in front of this space has been postulated to give a reasonable account for the generation of the outer border frog skin potential (Smith Martin and Huf 1968) The illustration gives preference to the Ussing and Windhager (1964) skin model over that proposed by Cereijido and Rotunno (1968) because it seems perhaps better substantiated by experimental facts Several locations for anion membrane interactions are indicated, lying in the Na pathway Whichever pathway one chooses the Na to take on its inward movement, this is not likely to be the same pathway for Na out-

flux, Cl influx and Cl outflux (see Discussion, section 2) This, in turn, would then limit the number of sites for anion-membrane interactions which must exist in the light of the data presented in this study

The author wishes to express his appreciation to Professor Dr H H Ussing and his staff for most cordial and helpful support of this work carried out during his sabbatical year in Copenhagen

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[illegible] he frog (Rana pipiens) *J ultra* [illegible]
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Acta physiol. scand. 1972, 84 382—395

From the Department of Medical Physiology C, University of Copenhagen Denmark

Gas Permeability of the Alveolo-pleural Wall in the Mouse Lung

By

Birgit Andersen and Joop Madsen

Received 22 September 1971

Abstract

Andersen B and J Madsen *Gas permeability of the alveolo pleural wall in the mouse lung* Acta physiol scand 1972 *84* 382—395

Transpulmonary pressure was recorded in isolated mouse lungs during and after step increases in volume The concomitant sudden increase in transpulmonary pressure was immediately followed by a sharp pressure fall indicative of stress relaxation This fall passed into a less [illegible] tude In these experiments diffusion capacities for N_2 between 0 1 and 0 2 μl min^{-1} cm H_2O^{-1} were found The volumes of gas disappearing from the lungs per unit time and pressure were slightly larger when the experiments were conducted in air than when done in N_2 This difference is probably due mainly to oxygen consumption by surviving lung tissue Ne passed the alveolo-pleural wall at a rate 3 times that of N_2 while O_2 passed the wall at a rate twice that of N_2 These figures are consistent with gas diffusion through the intact lung wall

In pressure-volume studies of lungs it is usually taken for granted that the lungs behave like air tight bags and that reliable constant pressure volume readings can be obtained after elastic equilibrium is achieved (Beckman and Weiss 1969 Gribetz Frank and Avery 1959 Mead Whittenberger and Radford 1957 Wohl Turner and Mean 1968, Wright 1968) When leaking is observed it is regarded as an artifact (Beckman and Weiss 1969)

During a study of the possible effects of liquid fluorocarbon breathing on the static compliance of mouse lungs we consistently found that the lungs leaked i e the transpulmonary pressure continued to fall after injection into the lung of a volume of air The present experiments were undertaken to describe this pressure fall quantitatively and to elucidate its nature

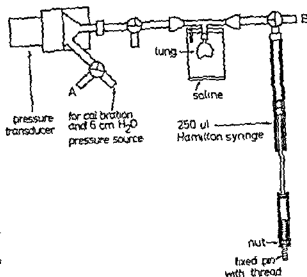


Fig 1 Schematic illustration of apparatus used for study of pressure fall in isolated mouse lungs in air See method section for details

Methods

Preparation of animals

Male mice (NMRI strain) were killed ... in i p nembutal-haloanisone* ... prevent collaps of the lungs sure corresponding to 6 cm H ... preliminary experiments the intrapleural pressure was found when the thorax was opened ... to be −6 cm H_2O)

The anterior thoracic wall was removed and the lungs were lifted out of the thorax by the tracheal cannula while pleural and vascular attachments to the carcass were cut with fine scissors These manipulations were done with the utmost care and contact between lung surface and instruments was avoided as far as possible The heart remained appendent to the lung preparation

The lungs were then transferred to the apparatus described below During the transfer the tracheal cannula was clamped, and the pressure in the apparatus was raised to 6 cm H O before connection between lungs and apparatus was established The initial lung volume thus obtained was supposed to be the same as the functional residual volume

Experiments on pressure fall in air

Pressure changes after a volume increase were studied in the apparatus shown in Fig 1 The lung was mounted on the vertical leg of a T tube Via 3 way stopcocks it was connected to a P23BB Statham pressure transducer and to a 250 µl gastight H... was filled with air its inner volume be... open to the atmosphere ... apparatus was temperat... ... in a number of ex of the apparatus The ... periments the temperatu... ... the isolation was controlled by mean of a Mettler TM 15 electrical thermometer

By mean of the Hamilton syringe air was injected into the lungs A volume of 50 µl was usually displaced from the syringe at a time The pressure difference between system and surrounding atmosphere (equal to the transpulmonary pressure) was continuously recorded

* Haloanisone Janssen Fluanisonum 10 mg acid lactic 9 mg methylparahydroxybenzoic 0 5 mg propylparahydroxybenzoic 0 05 mg aqua dest ad 1 ml 1 µl Haloanisone + 1 µl Nembutal 6 % were injected i p per g b w

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From the Department of Medical Physiology C, University of Copenhagen, Denmark

Gas Permeability of the Alveolo-pleural Wall in the Mouse Lung

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Received 22 September 1971

Abstract

ANDERSEN, B. and J. MADSEN. *Gas permeability of the alveolo-pleural wall in the mouse lung.* Acta physiol. scand. 1972. *84.* 382—395.

Transpulmonary pressure was recorded in isolated mouse lungs during and after step increases in volume. The concomitant sudden increase in transpulmonary pressure was immediately followed by a sharp pressure fall indicative of stress relaxation. This fall passed into a less pronounced continuing decline which appeared to decrease monoexponentially with time. Time constants for this monoexponential pressure fall scattered between 0.005 and 0.017 min^{-1} with a tendency to highest values in small animals. In similar experiments, where the lungs were filled and surrounded with N_2, time constants were found of the same order of magnitude. In these experiments, diffusion capacities for N_2 between 0.1 and 0.2 $\mu l\ min^{-1}\ cm\ H_2O^{-1}$ were found. The volumes of gas disappearing from the lungs per unit time and pressure were slightly larger when the experiments were conducted in air than when done in N_2. This difference is probably due mainly to oxygen consumption by surviving lung tissue. Ne passed the alveolo-pleural wall at a rate 3 times that of N_2, while O_2 passed the wall at a rate twice that of N_2. These figures are consistent with gas diffusion through the intact lung wall.

In pressure volume studies of lungs it is usually taken for granted that the lungs behave like air-tight bags and that reliable "constant" pressure-volume readings can be obtained after elastic equilibrium is achieved (Beckman and Weiss 1969, Gribetz, Frank and Avery 1959, Mead, Whittenberger and Radford 1957, Wohl, Turner and Mead 1968, Wright 1968). When leaking is observed, it is regarded as an artifact (Beckman and Weiss 1969).

During a study of the possible effects of liquid fluorocarbon breathing on the static compliance of mouse lungs we consistently found that the lungs "leaked", i.e. the transpulmonary pressure continued to fall after injection into the lung of a volume of air. The present experiments were undertaken to describe this pressure fall quantitatively and to elucidate its nature.

Experiments with lungs in situ

A number of experiments were conducted with the lungs in situ In these experiments the anim ls were k lled

constancy of the carcass was controlled during the pressure recording

Comparison of permeability for different gasses

Theory Whether the ... mechanical porosity ...) may be elucidated by ... v molecular weight and ... weight and high tissue solubility (Xe)

In the case of gross mechanical lesion gas will pass through the membrane by "bulk flow" and no difference would be expected between the rates of escape for different gasses However the rate would depend on the magnitude of the lesion

If the leak is of a porous nature, the ratio between diffusion capacities for N_2 and Xe will be the reciprocal of the ratio between the square roots of their molecular weights

$$\frac{DN_2}{DXe} = \frac{\sqrt{133}}{\sqrt{28}} = \frac{2\ 18}{1}$$

...ated to the square of the molecular weights

Consequently the ratio between diffusion capacities for N_2 and X would be

$$\frac{DN_2}{DXe} = \frac{\sqrt{133}}{\sqrt{28}} \times \frac{0\ 0147}{0\ 099} = \frac{1}{3\ 24}$$

for a membrane with the solubility characteristics of water (Linke and Seidell 1965)

In our experimental setup we also had the opportunity to compare the diffusion capacities for N_2 and O_2 In case of a mechanical porosity their ratio would be expected to be

$$\frac{DN_2}{DO_2} = \frac{\sqrt{32}}{\sqrt{28}} = \frac{1\ 07}{1}$$

If the diffusion barrier is an intact membrane with aqueous solubility properties (Linke and Seidell 1965) this ratio should be

$$\frac{DN_2}{DO_2} = \frac{\sqrt{32}}{\sqrt{28}} \times \frac{0\ 0147}{0\ 0286} = \frac{1}{1\ 83}$$

Experimental The expe... was removed ... of a low dead ... E and F Th... connected to ... recorder D ... G (LKB Var... into the bure... spheric pressure When B was in position for e...ration gas w... out through the water in M (giving a back pressure of 5 cm H_2O) ... was col... the buret I During expiration the left pump G removed mercu... fr... lung was above 5 cm of water Thus by switching B ... expiration the lung could alternatively be inflated to ... a pressure

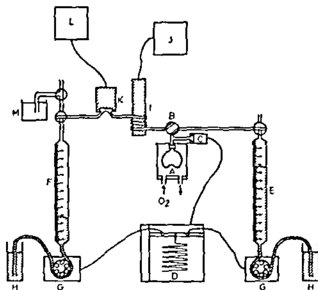


Fig 3 Experimental setup for examination of gas permeability of the mouse lung A Lung in glass chamber, B Stopcock, C Pressure transducer, D Recorder with microswitches steering pumps G (relays not shown) E Gas-burette delivering gas to lung (water jacket and arrangement for filling burette not shown) F Gas-burette for collection of gas from lung (water jacket not shown), G Peristaltic pumps H Mercury reservoirs, I Scintillation detector, J Meditronic differential ratemeter and scaler, K Oxygen electrode L Radiometer pH meter 27 and gas monitor PHA 927 b The electrical circuitry is not shown in detail

of 5 cm H_2O In 7 expts the time for such a cycle was 10 s, in 2 expts 30 s and in 1 expts. 2 min

Before the experiment the burette E was filled with humidified nitrogen containing a trace amount of Xe^{133}* The gas mixture was analyzed for contamination with oxygen and its Xe^{133} activity was measured This was done by passing it through a glass capillary wound around a scintillation detector I (Meditronic SD223) The gas was led from the burette to the detector via a short circuit not shown on the figure

During the experiment the external surface of the lung was continuously flushed with water saturated oxygen at a rate of at least 2 l/min The expired gas passed to the detector I as shown in the figure and from that to an oxygen electrode K (Radiometer E5046)

Expiratory gas was released through M until constant values for radioactivity and oxygen tension were obtained This would usually be the case when 8—9 ml of gas had passed through the lung At this point collection in the burette F was commenced The collection was continued until 12 15 m had been accumulated The experiment was then stopped Inspired and expired volumes were read at the burettes and corrected to standard conditions The expired gas was analyzed in duplicate for oxygen and for traces of CO_2 in the micro Scholander apparatus (Scholander 1947) The average radioactivity of the expired gas was calculated for the collection period

Calculations The net amounts of nitrogen and oxygen leaving or entering through the pulmonary surface were calculated from the volumes and compositions of the "inspired and "expired gas-mixtures The net loss of Xe^{133} through the pulmonary surface was expressed as the percentage of inspired Xe^{133} that disappeared from the lung Calculation of Xe volumes could not be done since no information was available on the specific activity of purchased Xe^{133}

Comparison of diffusion capacities for N_2 and Xe Since the tensions of N_2 and Xe on the outside of the lung were virtually zero their driving pressures for diffusion through the lung wall would equal their partial pressures inside As the amount of a gas diffusing through a barrier is proportional to the driving pressure
the barrier must be independent of the driv
the ratio between the fractions of inspired
of the diffusion capacities (μl gas/mm Hg/r

$$\frac{DN_2}{DXe} = \frac{(\text{vol } N_2 \text{ inspired} - \text{vol } N_2 \text{ expired}) \times \text{c.p.m. inspired}^{**}}{\text{vol. } N_2 \text{ inpired} \times (\text{c.p.m. inspired} - \text{c.p.m. expired})}$$

* According to its activity 0 5—2 ml of the gas was added to 60 ml of N_2 The purchased Xe contains about 1/4 Xe the rest is air

** c.p.m expired were calculated with correction for volume change during passage of lung

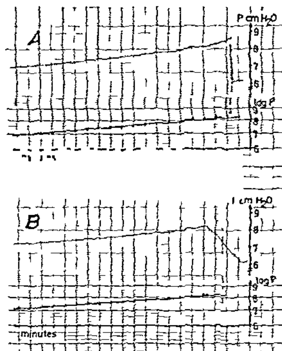


Fig 4 A Variations in transpulmonary pressure during and after fast injection into lung preparation of 70 μl of air Below the same values are given in a semilogarithmic diagram with extrapolation of the curve to midinjection time Weight of mouse 33 gs B Same after slow injection of equal volume of air

Dead sp . . . d ... are assumed to affect Ne and Xe to ... DN2 DXe ratio This assump-

...essure gradients for N2 and O ... therefore the ratio of diffusion capacities for N_2 and O_2 is estimated as the ratio between the volume of N_2 that has escaped through the alveolo-pleural wall and the volume of oxygen that has entered over the same barrier

$$\frac{DN_2}{DO_2} = \frac{\text{vol of } N_2 \text{ lost during the experiment}}{\text{vol of } O_2 \text{ gained during the experiment.}}$$

Since the *intrapulmonary* pressure at an average is 10 mm Hg above the extrapulmonary pressure ... han the oxygen gradient However this d f ... en or oxygen gradients ... ventilation of the lung

Results

Pressure fall studied in air

When 50 μl of air are displaced from the Hamilton syringe in about 5 s the transpulmonary pressure increases steeply as shown in Fig 4 A Immediately after the displacement the pressure starts falling The pressure fall may be separated in 2 components a primary steeper drop lasting 2—3 min and a secondary protracted decline which with good approximation is an exponential function of time (Fig 4 A)

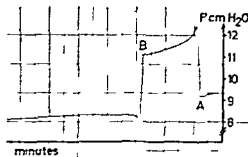


Fig 5 Stress relaxation (A) and stress recovery (B) in a mouse lung after fast injection or removal of 70 µl of air

Initial pressure fall The initial pressure fall can be reduced or avoided if the air is injected slowly into the lungs using 3 min for the injection instead of 5 s (Fig 4 B) This procedure apparently does not influence the protracted, exponential fall, and by extrapolation to midinjection time the same pressure is obtained by the two methods

A sudden removal of 50 µl of air from the lung results in a pressure drop which is immediately followed by an increase in pressure This increase wears off and is followed by a slow decline (Fig 5)

Secondary pressure fall The rate of the secondary exponential fall in transpulmonary pressure can be expressed as a k value giving the fractional decrement in pressure per minute This figure was found in the order of magnitude of 0 01 min⁻¹ For each preparation the k value was calculated as the average of 2—3 consecutive determinations The standard deviation for these single determinations was at an average 0 0018 min⁻¹

The 2nd and 3rd injections of air into the lungs were done before the transpulmonary pressure reached the preinjection level Thus k values were determined at transpulmonary pressures ranging from 6 to 14 cm H_2O No correlation was found between k values and the transpulmonary pressures around which they were determined

The k values were however found to depend on the weight of the animal as shown in Fig 6 A regression line calculated for these values has the formula

$$k = -0\,00029 \times \text{weight} + 0\,0177 \qquad (r = 0\,74)$$

Pressure fall studied in N_2

The results of these experiments are shown in Fig 6 and in Table I As seen from Fig. 6 the k values were found within the range of k values for air filled lungs from mice of similar weight The average k value found in the N_2 studies was 0 0087 min⁻¹ (SD 0 0024) The average weight of these mice was 28 g The average k value for large and medium sized lungs studied in air was 0 0092 min⁻¹ (SD 0 0024) The average weight of these mice was 29 g It is seen that the two groups are comparable on a weight basis

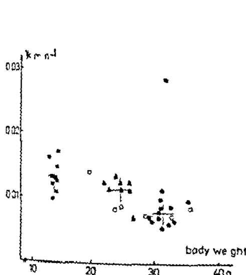


Fig 6

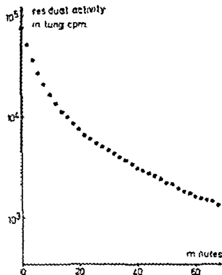


Fig 7

Fig 6 k values plotted as function of body weight for 30 mice in 3 weight groups (black symbols) Horizontal and vertical lines cross in the average for each group and represent ± 1 standard deviation The point in brackets has not been included in the calculations This very high k value is supposedly caused by an artificial leak The open symbols indicate corresponding k values and body weights for experiments conducted in N_2

Fig 7 Disappearance of Xe^{133} from mouse lungs with closed trachea

In Table I is calculated diffusion capacities for N_2 for the alveolo-pleural wall The figures range from 0 1 to 0 2 μl N_2 min^{-1} $cm\ H_2O^{-1}$ The variation may be greater than suggested by these figures since the diffusion capacity would be expected to increase with the weight of the animal a correlation which is not apparent from the present data

Pressure volume relationships

The pressure increase after injection of 50 μl of N_2 is shown in Table I From these figures an average compliance value of 17 6 μl $cm\ H_2O^{-1}$ SD 4 1 * can be calculated The corresponding figure for the combined large and medium sized lungs studied with air is 30 4 $\mu l/cm\ H_2O$ (SD 7 9 * The difference between the two groups is very significant ($p < 0.001$)

Preliminary experiments in which static compliance of mouse lungs was first determined in air later in N_2 seems to indicate a reduction in compliance when the lungs are ventilated in an oxygen free medium

* These values are not corrected for the volume of gas used to increase pressure in the recording system The true pulmonary compliances are 3 5 $\mu l/cm\ H_2O$ lower than indicated above

Discussion

The fall in transpulmonary pressure seen in the mouse lung after a step increase in volume may be caused a by the streatching of lung components b by a loss of gas due to leakage or diffusion through the lung wall, c by the opening of collapsed alveoli or d by a consumition of oxygen by surviving tissues

Initial pressure fall after a step increase in volume or pressure recovery after step emptying has been described in lungs from humans (Gribetz *et al* 1959 Marshall and Widdicombe 1960, McIlroy 1952, Sharp *et al* 1967), cats (Hildebrandt 1969), rabbits (Hughes *et al* 1959) and rats (Clements *et al* 1961)

It is demonstrated that the mouse lung presents stress recovery after a sudden reduction of volume Consequently, stress relaxation must be considered the most likely explanation of the initial pressure fall found after volume increase in the present experiments The explanation is supported by the demonstration that the initial pressure fall can be diminished or avoided if the lung is distended at a slow rate The finding of identical pressures by extrapolation to midinjection time in curves with and without a distinct initial fall makes it further unlikely that a gas leak should give rise to the initial fall

The viscous behaviour of the lung may be caused by properties of the alveolar surfactant as suggested by Mead *et al* (1957) who found the hysteresis of volume pressure curves much reduced, when lungs were filled with saline Alternatively, the viscosity may be located in tissue elements as demonstrated in the rabbit by Hughes *et al* (1959) The authors have observed distinct initial pressure falls in mouse lungs filled with saline (unpublished observation) suggesting that at least the second mechanism is at work in the mouse lung Since the present experiments were conducted at volumes over the functional residual capacity an opening of alveoli seems unlikely as explanation of the initial fall

The following protracted pressure fall is connected with a loss of gas as shown by the disappearance of Xe^{133} from isolated lungs and from lungs *in situ* This is also apparent from the experiments on lungs filled with and surrounded by nitrogen

However a comparison of the air filled and the nitrogen filled lungs shows that a loss of gas through the alveolo pleural wall can hardly be the only mechanism involved in the slow pressure fall in air filled lungs The volume of air lost from the lung per minute per unit pressure difference may be calculated as the product of k and $\frac{\Delta V}{\Delta P}$ for (lung+measuring system) The average value for medium sized and large, air filled lungs is found to be 0 27 μl air min^{-1} cm H_2O^{-1} (SD 0 06) The corresponding average figure ($\sim$ the diffusion capacity) for nitrogen filled lungs is 0 15 μl N_2 min^{-1} cm H_2O^{-1} (SD 0 04) The difference between the volumes of gas disappearing from the lungs in air and in N_2 experiments is most likely due to oxygen consumption by the lung tissue

Barron *et al* (1947) have reported O_2 uptakes by rat lung slices amounting to 7 7 μl O_2 per hour per mg dry weight at a respiratory quotient of 0 85 Figures of

the same magnitude were found for rabbits and guinea pigs. If these figures can be applied to the intact, isolated mouse lung they would correspond to an oxygen consumption of about 20—25 μl for a pair of lungs per minute. For comparison with our experiments this figure should be reduced to 5—10 μl per minute, since our experiments were conducted at temperatures about 15° C below Barrons. Still this figure is very great compared to the difference between the volumes of gas from air filled and from N_2 filled lungs (about 1 μl per minute at a transpulmonary pressure of 10 cm H_2O). Thus the pressure fall in the isolated, air filled lung appears to be the resultant of a number of factors: 1) loss of nitrogen through the lung wall; 2) metabolic consumption of oxygen, 3) loss of CO_2 from the lung and probably 4) influx of O_2 from the surroundings to partial replacement of metabolically used O_2. The data reported here do not permit a quantitative evaluation of the factors 2, 3 and 4. On this complex background it is surprising that the pressure falls after injection of air are so similar to those seen after injection of N_2.

The results of the N_2-Xe-O_2 experiments indicate that the passage of gases across the alveolo-pleural wall cannot be explained by a mechanical leak. The findings are consistent with diffusion of the gasses through an intact barrier of aqueous solubility characteristics. The thickness of the alveolo-pleural partition (L) can be evaluated from the N_2-diffusion capacities reported here if it is treated as an aqueous membrane. The free surface area (A) of the lungs from a 25—30 g mouse can be estimated to about 5 cm² at an intrapulmonary pressure of 8 cm H_2O (calculated as the area of a cone with the same height and a basal diameter as the average of the frontal and saggital maximum measures of the preparation). The diffusion coefficient (Δ) for N_2 in water at 25° C is about 2×10^{-5} cm² sec^{-1} (Washburn 1929). The absorption coefficient (α) for N_2 in water at 25° C is 0.015 (Linke and Seidell 1965). The average diffusion capacity found here is 0.15 μl N_2 cm H_2O^{-1} min^{-1}. Using these values and a modified Fick's 1. law

$$V_{gas} = \Delta \cdot A \cdot \frac{1}{L} \cdot \alpha \, (P_1 - P_2),$$

where $P_1 - P_2$ is the tension difference over the membrane. L is found to be 6 μ. Since A is probably underestimated (the preparation is not really a cone) L is underestimated too. However its magnitude fits well with microscopic observations giving values between 2 and 20 μ (unpublished observation). The high values may be caused by interalveolar septa lying in the plane of the cut.

The diffusion capacities found in the N_2 experiments cannot be compared with similar values from the N_2—Xe—O_2 experiments since the gas composition and thus the N_2-tension in the alveoles bordering the lung wall is unknown in the latter experiments. However, by assuming similar diffusion capacities for N_2 in the two series the N_2-tension on the inside of the alveolo pleural wall can be calculated. If this is done with the average diffusion capacity (0.15 μl N_2 cm H_2O^{-1} min^{-1}) and the average N_2 loss per minute for the mice F through M in the N_2—Xe—O_2 series (34 μl/min) the "inside" P_{N_2} is found to 227 cm H_2O. This figure indicates a considerable

'functional dead space' (dead space of stopcock, anatomical dead space and centrally located alveoli) in these experiments where gas exchange only occurs through the pleural surface of the lung.

The finding of a measurable gas permeability over the alveolo-pleural wall is not surprising. Permeability of the alveolo-capillary wall is a prerequisite for its physiological function, and gas permeability of the serous lining is a condition for the resorption of gas from the pleural cavity. The following circumstances may explain why it has not been noticed in other studies on isolated lungs.

The fractional decrease in transpulmonary pressure per time unit caused by N_2 loss through the lung wall is most likely directly related to the surface area through which N_2 is lost, and inversely related to the volume of gas in the lungs, everything else being equal. Since a certain increase in lung volume is accompanied by an area increase that is smaller ($A \sim \sqrt[3]{V^2}$), the importance of the described permeability will decrease when the size of the lungs increase. If, for example, the volume of the mouse lung was increased 2000 times (corresponding to the weight ratio man/mouse), the area would increase only $\sqrt[3]{2000^2} = 1.6 \times 10^2$ times and a corresponding k value would change from for example 0.01 to 8×10^{-4}, more than a 10 fold reduction. Further it is well known that the oxygen consumption per weight unit tissue decreases markedly with increasing size of the animal, while the oxygen stores of the lung probably increase proportionally to the weight.

These factors may well make the slow pressure fall inconspicuous and insignificant in larger animals. In small animals, however, this effect must be taken into account when pressure-volume studies are conducted. We correct for this leak by plotting the pressure as a function of time in a semilogarithmic system and extrapolate the linear part of the curve to midinjection time. The difference between this extrapolated value and the preinjection pressure is then taken as the pressure increment to which volume change is related.

The authors thank professor P. Kruhøffer, dr. Niels A. Lassen and dr. P. Sejersen for valuable discussions and suggestions. Mrs. Karen Soholm has contributed with skillful technical assistance. The work was supported by Carl og Ellen Hertz's legat til dansk læge- og naturvidenskab, Frk. P. A. Brandt's legat til fremme af fysiologisk-patologisk forskning og fysiologisk-gymnastisk videnskab, and Løvens kemiske fabrik's legat.

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Methods

Cats were kept in l
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Catheterisat of a liver vein was performed via the external jugular vein and right heart
the catheter

Temperature regulation and cooling

In the control period the body temperature was kept between 38 and 38.5° C. Cooling was performed ted with an alcohol solution ithin 30 min. The tempera- The body temperature was lower oesophagus. In some control temperature after the cooling period and in some the temperature was lowered stepwise.

Administration of taurocholate, Indocyanine Green and ethanol

To compensate for the enterohepatic circulation of bile acids taurocholate sodium (Michrome) was infused sufficient to maintain a holate used for infusions a priming dose of 300

continuously (36 µmol)/kg/n
arterial concentrations at which the elim tion rate was therefore, within wide limits, independent of concentration and hepatic blood flow (Larsen 1963). The priming dose of ICG and ethanol was dissolved in 20 ml isotonic NaCl. Taurocholate, ICG and ethanol for continuous infusion were dissolved in 50 ml isotonic NaCl which was infused at rates of 0.1 ml/min. To increase the stability of the dye solution albumin was added and the infusion was protected against light.

Analytical procedures

The bile flow was measured gravimetrically without correction for the specific weight of bile which was found to be 1.02 ± 0.008 (SD). Bile samples for determination of ICG were added to equal volumes of 10 % albumin and diluted 1 : 250 with 0.9 % NaCl. A standard curve was prepared in each experiment from bile collected before the infusion was started. The samples were read against a diluted blank bile albumin mixture in a Zeiss spectrophotometer at 790 nm, the absorption maximum for ICG in bile. Sodium and potassium in the bile were determined by flame photometry, chloride by potentiometric titration and total CO_2 by the microdiffusion method of Conway. Bicarbonate was calculated from the total CO_2 and pH using the Henderson Hasselbalch equation assuming a pK_1 of 6.1. Total bile acids were determined enzymatically as described by Bruusgaard (1970) without chromatographic purification. In our laboratory this method yielded a recovery of 94 % with a coefficient of variation of 3 % on different concentrations of desoxycholic acid.

Blood samples for determination of ICG were centrifuged at 3000 rpm for 30 min. The plasma was diluted 1 : 25 with a 5 % albumin solution and read at 800 nm in a Zeiss spectrophotometer. Standard preparations of ICG in a 5 % albumin solution were added to 4 volumes of blank plasma for construction of standard curves. The was corrected for blank density by the method of Nielsen (1963). Ethanol was determined enzymatically as described by Larsen (1963).

Calculations

The biliary output of both ICG and bile acids was calculated as the product of the bile flow and the biliary concentration of ICG and bile acid respectively. Assuming a steady liver content of bile acids the difference between the amount of bile acids excreted and the amount infused was taken as a measure of the endogenously produced bile acids. Plasma clearance of ICG was calculated as the amount eliminated divided by the arterial plasma concentration a

equilibrium In some of the cats an equilibrium concentration was not obtained after cooling In these cases clearance was calculated as the amount of ICG infused subtracted by the amount retained in the plasma volume (5 % of b w) per minute divided by the mean concentration in the cooling period The hepatic extraction ratio of the dye was calculated as the arterio-hepatic venous difference divided by the arterial concentration at equilibrium or the mean concentration when equilibrium was not obtained The estimated hepatic blood flow (EHBF) wa [illegible]
[illegible] ure
of [illegible] ese
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hepatic as well as the extra hepatic elimination was calculated from the amount of ethanol infused per minute corrected for the amount retained in or removed from the solvent space as described by Larsen (1963) The hepatic elimination of ethanol was determined by multiplying the EHBF by the arterio-hepatic venous ethanol difference

Statistical procedures

The significance levels were determined by the method of paired comparisons using the Student's t test Correlation was carried out according to Sokal and Rohlf (1969)

Results

One experiment in which the body temperature was lowered in steps is shown in Fig 1 and 2 In Fig 1 it is seen that the bile flow remains constant during cooling until the temperature passes 37 5° C It then suddenly decreases (23 %) further cooling being without additional effect The biliary concentration of ICG is essentially constant in the control period After cooling there is a slight decrease in concentration followed by a steady increase during the rest of the experiment The biliary excretion of ICG in the control period is equal to the amount of ICG infused After cooling the excretion decreases to 75 % of the infused ICG and then returns slowly towards the control level Further cooling produced little or no effect In Fig 2 it is seen that the ICG concentrations in both arterial and hepatic venous plasma are elevated after cooling but the arterio hepatic venous difference is not altered significantly indicating that there was no significant change in the hepatic blood flow Apparently the ICG elimination can now only be maintained at a higher concentration which is reflected in a decrease of the plasma clearance of the dye (21 %) and the extraction ratio (25 %) The change in the slope of the ethanol curve corresponds to a 24 % decrease in the elimination rate Neither the ICG uptake nor the elimination rate of ethanol are further decreased by cooling to 36° C

The results from 16 cats which were cooled 2° C from a control level of 38 or 38 5° C are listed in Tables I—IV In all experiments except one (Table I), cooling caused a decrease in the bile flow (mean 23 %) and the total elimination rate of ethanol (mean 26 %)

A significant decrease of clearance (mean 26 % and extraction ratio (mean 19 %) was found, whereas the 9 % decrease in estimated hepatic blood flow was not significant The correlation between the relative decrease in ethanol elimination and bile flow is shown in Fig 3 The correlation coefficient was 0 78 which is statistically significant ($p < 0\ 005$)

From Table II it appears that the correlation between the total and the hepatic

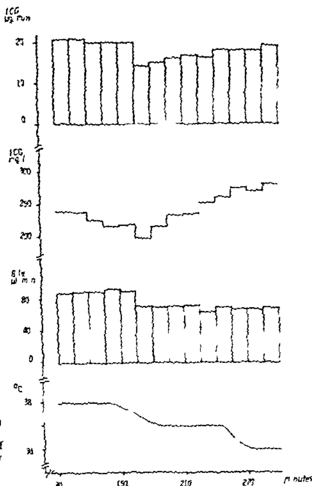


Fig 3 The effect of cooling on bile flow, the biliary concentration and the biliary excretion of *Indocyanine Green*. The lower curve shows the body temperature

TABLE I The results from experiments in which the bile flow, the hepatic uptake of Indocyanine Green and the elimination rate of *ethanol* were measured at normal body temperature and after cooling 1—2 C

	Weight kg	Bile flow µl/kg min		Elimination of ethanol µMol kg min		Extraction ratio of ICG per cent		Plasma clearance of ICG ml kg min		Estimated hepatic blood flow ml kg min	
		A	B	A	B	A	B	A	B	A	B
mean	3 2	16 3	11 8	35	26	30	25	6 6	4 9	37	34
sd	0 4	5 1	4 3	3 5	5 1	8 8	8 7	1 5	1 4	8 6	11 6
n	16	16		12		16		16		16	
		p < 0 001		p < 0 001		p < 0 001		p < 0 001		0 20	p 0 30

A = control period B = cooling period

TABLE IV The biliary bile acid concentration and excretion at normal temperature and after cooling 1—2° C

	Bile flow µl/min		Bile acids mM		Excretion of bile acids µmole/min		Excretion of endogeneous bile acids µmole/min	
Cat No	A	B	A	B	A	B	A	B
4	36 7	28 7	31	37	1 14	1 06	0 50	0 42
13	42 6	38 9	33	35	1 41	1 36	0 75	0 70
15	48 7	38 3	39	44	1 90	1 69	1 36	1 15
16	51 0	35 6	24	29	1 22	1 03	0 54	0 35

TABLE V The results from experiments in which the bile flow was measured at different temperatures

Bile Flow µl/kg/min			
Cat No	38 5° C	36 5° C	38 5° C
17	7 6	5 3	7 8
18	12 7	8 0	12 5
19	12 4	8 1	9 7
20	7 2	4 8	7 8
mean	10 0	6 6	9 5
	38 5° C	37 0 C	35 5° C
21	22 7	17 4	16 1
22	14 8	9 4	7 4
23	11 8	8 4	7 4
24	9 5	6 5	6 9
mean	14 7	10 4	9 5

between inorganic cations and anions increased From Table IV it appears that the bile acid excretion was slightly reduced after cooling, although the biliary concentration of bile acids had increased In seven of the cats the bile flow was followed for 45 min prior to the infusion of taurocholate and a value of 10 2 µl/kg/min was found In these cats infusion of taurocholate increased the bile flow by 25 %

The bile flow and electrolyte composition the ICG clearance and extraction ratio, as well as the estimated hepatic blood flow were of the same range in cats which were only given taurocholate and ICG as in those which were also infused with ethanol Neither was there any difference in the effect of cooling between the two groups

In eight cats in which a continuous infusion of taurocholate was given the reversibility of the effect of cooling and the effect of stepwise cooling was investigated The results are listed in Table V The effect is reversible and is initiated at a temperature between 37 and 38° C Further cooling has only a slight effect on the bile flow

Discussion

The present results have confirmed the effect of slight hypothermia on the elimination rate of ethanol and the hepatic uptake of ICG (Larsen 1971) Although the effect was smaller than previously observed it still exhibited the character of an all-or none reversible reaction The reaction was elicited at a body temperature of about 37° C and further cooling was without significant effect The results have furthermore revealed that cooling affects the hepatic excretory function in a way which corresponds closely with the changes in the elimination rate of ethanol and the hepatic uptake of ICG

Secretion and composition of bile

The spontaneous bile flow in the present series is about 50 % higher than found in cats by Scratcherd (1965) This discrepancy may, however, be explained by the difference in anesthesia employed Previous studies of the effect of hypothermia on the secretion of bile have been mainly concerned with temperatures far below the physiological level and in consequence with greater temperature intervals In rats the bile flow decreased with a Q_{10} of 1 2—2 0 (Archdeacon, Danforth, and Dummit 1954, Kalow 1950) whereas Kjellgren (1954) has shown that the bile flow in rabbits decreases in parallel with temperature, when the liver was cooled 5° C, by means of intra abdominal cooling, the bile flow was halved Brauer (1954) found Q_{10}-values of the bile flow in perfused rat livers which varied between 2 8 and 5 5 depending on the temperature interval investigated Similar results were obtained by Morris (1960) and Vanlerenberghe (1965) One figure given by Brauer Holloway, and Leong 1957, (Fig 1) indicates that the effect of temperature was initiated in an all-or none fashion

It is generally accepted that the bile is composed of a hepatocellular or canalicular fraction and a ductular fraction as recently reviewed by Brooks (1969) The hepatocellular fraction depends partly on an active secretion of bile acids and partly on an active secretion of inorganic cations (Wheeler Roos and Bradley 1968, Erlinger *et al* 1970) The ductular fraction is dependent on an active secretion of bicarbonate which may be stimulated by secretin. In the cat the biliary concentration of bicarbonate is only slightly higher than that in plasma (Scratcherd 1965) indicating that in the cat the ductular secretion of bile is of minor importance The observed decrease in bile flow caused by cooling is therefore most probably caused by a reduction of the hepatocellular fraction

The electrolyte composition of the bile does not differ from the findings of Scratcherd (1965 except for a higher sodium concentration The composition of bile in hypothermia was studied by Fisher *et al* (1956) in dogs They found that the biliary concentration of cholic acid was not changed upon cooling although the bile flow decreased indicating that the choleretic action of bile acid was not changed This is in contrast to the results of Kalow (1950) and Vanlerenberghe (1965) who found a reduction in the choleretic action of a single dose of dehydro-

cholate in hypothermia In the present experiments the fall in bile flow upon cooling was accompanied by an increase in bile acid concentration supporting the findings of Kalow (1950) and Vanlerenberghe (1965) The total excretion of bile acids is not reduced to the same extent as the bile flow in contrast to the calculated excretion of endogenously produced bile acids The increase in bile acid concentration during cooling is in accordance with the observed increase in the difference in concentration between biliary inorganic cations and anions

In summary, cooling apparently affects the hepatic excretory function causing a permanent decrease in bile flow accompanied by a slight increase in bile acid concentration The effect on bile flow may partly be due to the decrease in the bile acid excretion However a decrease in bile flow accompanied by an increase of the bile acid concentration means either a diminished choleretic action of bile acids or a reduction of a non bile acid dependent bile fraction If a ductular fraction is neglected, the latter might be the electrolyte secretion dependent fraction described by Wheeler *et al* (1968) and Erlinger *et al* (1970) The effect of cooling may be explained merely as an overall effect of temperature on liver cell metabolism but the all-or none reaction to cooling supports the view that the changes in bile flow may be due to a reduction in the number of perfused sinusoids (Brauer, Leong and Holloway 1954 Morris 1960) It should be mentioned that changes in portal and hepatic venous pressure may be of importance for the bile secretion but these pressures were not measured in the experiments

Hepatic uptake and excretion of ICG

The recovery of ICG in the bile was 94 % in the control period which agrees well with a recovery close to 100 % found in dogs (Wheeler Cranston and Meltzer 1958 Ketterer Wiegand and Rapaport 1960) and rats (Hunton Bollman, and Hoffman 1960)

Few experiments describe the handling of dyes by the liver in hypothermia In rat liver slices Brauer and Pesotti (1949) found that the uptake of bromsulphalein (BSP) was almost independent of temperature In the intact dog Brokaw and Penrod (1949) showed that the disappearance rate of BSP after a single dose was far lower in dogs at 35° C compared with normothermic dogs but there was no difference between dogs at 35 C and 30° C These two studies indicate that the effect of hypothermia on the hepatic uptake of dye depends on vascular changes rather than a Q_{10} effect of temperature In a microscopic study in intact rats however, Hanzon (1952) found no change in the sinusoidal blood flow after cooling to 28° C whereas the hepatic uptake and excretion of uranine was depressed In the present experiments the arterial ICG concentration rose after cooling apparently without changes of EHBF This means that the rate constant for the ICG uptake has decreased, which may be due to changes in the metabolic activity of the liver cells It may however, also be explained by a reduction in the number of functioning liver cells, due to closure of some of the sinusoids As the hepatic blood flow

was nearly unaltered in the experiments this must involve an increase in flow through the remaining channels causing a decrease in the plasma clearance as well as the extraction ratio of the dye as found in the experiment. The decrease in the rate constant for the ICG uptake is compensated by the increase in the arterial ICG concentration, and the ICG uptake is normalized within approximately 30 min (Fig 2) In contrast the ICG excretion returns slowly towards the control level, which means that the excretory function is affected. This may also be due to either metabolic or intrahepatic vascular changes. A reduction in the amount of functioning liver tissue will cause a corresponding decrease in the excretion of ICG, but as the excretory capacity of the cells is not saturated, these may increase their excretion of the dye when a higher intracellular dye concentration is reached. This is in accordance with the model proposed for BSP excretion by [illegible] and [illegible] (1965)

The effect of cooling on the elimination of ethanol is therefore most probably due to an action on the liver, which — as was the case for the hepatic excretory function — may be of metabolic origin or due to vascular changes resulting in a decrease in the number of perfused sinusoids

Conclusion

It is possible that the metabolic processes underlying the elimination of ethanol and glycerol, the secretion of bile and the hepatic uptake and excretion of ICG are all equally sensitive to small changes in temperature However, the observed all-or none reaction to hypothermia of the different processes in question makes this effect less likely

It is therefore tempting to assume that hypothermia induces vascular changes in the liver with a reduction of the functioning liver tissue as a concequence This would explain why different functions of the liver are affected to almost the same relatively great extent as well as the all or none reaction to hypothermia

As discussed previously (Larsen 1971) this assumption is based upon the results from in vivo observations of the hepatic microcirculation (*e g* Knisely Block and Warner 1948 McCuskey 1966) In these studies it was observed that normal sinusoidal blood flow in a variety of species was intermittant and under the influence of different sfincter mechanisms However, intermittancy of sinusoidal blood flow has been denied by others (Maegraith 1958 Nakata, Leong and Brauer 1966) and its functional importance if any has never been demonstrated

Although the present results are not conclusive they do to some extent support the existence of uneven perfusion of liver sinusoids and they do demonstrate that even small changes in temperature probably within the limits of normal body temperature may be of importance for the regulation of liver function

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The Glomerular Filterability of Inulin and of Different Molecular Weight Preparations of Polyethylene Glycol in the Rabbit

By

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Abstract

Jorgensen, K. E., J. V. Moller and M. I. Sheikh *The glomerular filterability of inulin and of different molecular weight preparations of polyethylene glycol in the rabbit* Acta physiol scand. 1972 *84* 408—414

It has been claimed on the basis of clearance experiments that inulin is incompletely filterable through the glomerular membrane of the *rat kidney* (Berglund 1964, 1965) In the present study the renal excretion in the rabbit of inulin and different preparations of polyethylene glycol has been compared during constant infusion of these substances Identical clearance values were obtained for inulin and polyethylene glycol having molecular weights of 1500 (PEG—1500) and 4000 (PEG—4000) The ratio of the clearances between polyethylene glycol of a molecular weight of 6000 (PEG—6000) and inulin was smaller than unity (0.89 ± 0.03) Gel filtration analysis of plasma and urine samples indicated unrestricted filtration of inulin through the glomerular membrane of this species Furthermore gel filtration and ultrafiltration experiments indicated that the molecular size of inulin and PEG—1500 is similar while the molecular dimensions of PEG—6000 exceed that of the most high molecular weight fractions of inulin It is concluded that the clearance of inulin PEG—1500 and PEG—4000 may be used as a measure of the glomerular filtration rate in the rabbit whereas the glomerular filtration of PEG—6000 is restricted to some extent The incomplete filterability of PEG—6000 is attributed to the relatively larger molecular size

The use of inulin clearance as a measure of glomerular filtration rate in the kidney of various animal species presupposes that inulin is neither reabsorbed nor secreted by the tubular cells and that the glomerular membrane is freely permeable to the polyfructoside The results of micropuncture studies (Marsh and Frasier 1965 and Maude *et al* 1965) have shown that the tubules of the rat nephron are virtually impermeable to inulin However the view that inulin is freely filterable through the glomerular membrane (Hendrix Westfall and Richards 1936) has been challenged by Berglund (1965) who found that in the rat low molecular weight preparations of polyethylene glycol have a higher clearance than that of inulin On the basis of comparisons with the clearance values for sulfate (Berglund 1964) and mannitol (Berglund 1968) this author concludes that glomerular filtration of inulin is incomplete in the rat On the other hand it has been shown that in the dog the

clearance of inulin agrees with that of low molecular weight preparations of polyethylene glycol (Shaffer, Critchfield and Carpenter 1948), mannitol (Berglund 1968), and creatinine under free flow conditions (O Connell, Romeo and Mudge 1962), and there is thus no evidence for restricted glomerular filtration of inulin in this species

In previous studies on the renal excretion of urate in the rabbit (Møller 1965 a, b, 1966) we have used inulin and creatinine to measure the glomerular filtration rate, but the studies of Berglund raise the question whether there may also be objections to the use of inulin as a reference substance in this species We therefore decided to compare the excretion of inulin with that of different molecular weight preparations of polyethylene glycol in the rabbit The results of our study show that inulin and polyethylene glycol of a molecular weight of 1500 (PEG 1500) or 4000 (PEG-4000) are completely filterable through the glomerular membranes of the rabbit kidney, whereas polyethylene glycol of a molecular weight of 6000 (PEG 6000) has a smaller clearance than these compounds In agreement with these findings gel filtration experiments and ultrafiltration through cellophane membranes indicate that the molecular size of inulin is considerably smaller than that of PEG 6000

Methods

Animal Experiments The experiments were carried out on male rabbits weighing 2 5—4 0 kg The animals were anaesthetized with pentobarbital sodium (Nembutal Abbott) and the respiration maintained throughout the experiment by a respiration pump as previously described (Møller 1965 a) An external jugular vein and a carotid artery were cannulated for intravenous infusion and arterial blood sampling respectively and urine was collected from a urethral catheter A stock solution containing 1 % (w/v) inulin (Merck AG Darmstadt Germany) and 1 % (w/v) polyethylene glycol of a molecular weight of either 1500 or 4000 or 6000 (Fluka, Buchs Switzerland) dissolved in physiological saline was infused for 20 min following which it was replaced by an infusion fluid prepared by mixing equal volumes of the stock solution and physiological saline The infusion rate of both fluids was 2 0 ml/min

Collection of urine for clearance determinations was begun when the infusion had been given for 1 h Urine was collected for 6—12 periods with a duration of 10 min each Blood was drawn from the arterial catheter 2 min before the middle of the clearance period to correct for urinary dead space and at the end of each period the bladder was rinsed twice with 5 ml of physiological saline In some experiments the molecular weight dispersion of inulin in plasma and urine was estimated by gel filtration In these cases PEG was omitted from the infusion fluid and one urine sample was collected during the 2nd hour of infusion after which a large blood sample (20 ml) was drawn The sample of urine and plasma was chromatographed on a Sephadex [illegible]

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staltic pump and fraction [illegible] ultrorac fraction collector The distribution of the different substances in the gel phase (K_{av}) was calculated according to Laurent and Killander (1964) as

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

TABLE I Comparison between excretion of inulin and different preparations of polythylene glycol

Polyethylene Glycol	Number of Expts	Number of Periods	C_{In} (ml/min)	C_{PEG}/C_{In}	P_{In} (mg/ml)	P_{PEG}/P_{In}
PEG 1500	5	50	14 0	1 00±0 05	0 74	1 02±0 05
PEG 4000	5	30	13 3	1 02±0 01	0 80	1 01±0 01
PEG 6000	4	36	9 3	0 89±0 03	0 96	1 23±0 08

PEG 1500 PEG 4000 and PEG 6000 Polyethylene glycol of molecular weights about 1500 4000 and 6000 C_{PEG} Polyethylene glycol clearance P_{PEG} Plasma concentration of polyethylene glycol C_{In} Inulin clearance P_{In} Plasma concentrat on of inulin

in which V_e refers to the elution volume of the test substance and where V_o and V_t represent void volume and total volume respectively of the liquid phase of the column

Ultrafiltration Experiments The experiments were performed in the tubes described by Toribara Terepka and Dewey (1957) and the ultrafiltrate was collected after centrifuging at 1100 G for 1 hour from cellophane bags containing 4 ml of 0 1 % inulin or polyethylene glycol At the end of the experiment the concentration of inulin and polyethylene glycol in the ultrafiltrate and the bag content was measured as described below

Analytical Procedures Inulin in plasma urine and eluates from gel filtration was determined either by the resorcinol method of Schreiner (1950) or by the anthrone method of Van Handel (1967) as modified by Nixon (1969) The concentration of glucose in plasma samples was estimated according to Hagedorn Halstrom and Jensen (1946) to correct for the contribut on of glucose to the colour reaction for inulin Polyethylene glycol was estimated turbidimetrically by add tion of a solution containing trichloroacetic acid and $BaCl_2$ to cadmium filtrates of plasma and suitable dilut ons of urine as described by Hydén (1955)

Results and Discussion

nimal Experiments

summary of the results obtained by simultaneous determinations of the clearance values of inulin (C_{In}) and of different molecular weight preparations of poly etylene glycol (C_{PEG}) has been presented in Table I It is seen from the table that the clearance values of PEG 1500 and PEG-4000 are similar to those of inulin C_{PEG}/C_{In} not differing significantly from unity In contrast the clearance of PEG 6000 is smaller than inulin clearance C_{PEG}/C_{In} in this case being 0 89+0 03 Furthermore it appears from the table that there is no difference between the plasma concentration of PEG 1500 or PEG-4000 and of inulin This finding pre sumably indicates almost complete equilibration of the infused substances in the body fluids since polyethylene glycol and inulin were infused at the same concen tration On the other hand the plasma concentration of PEG 6000 was higher than that of inulin indicating retention of PEG 6000 because of a reduced renal excre tion of this substance

The molecular weight dispersion of inulin in plasma and urine samples has been examined by gel filtration and the results are shown in Fig 1 and 2 In these ex periments the gel filtration pattern of a plasma and urine sample from the experi mental animals was compared with that of inulin dissolved in plasma and urine collected from control animals in which inulin had not been infused It is apparent that there is no significant difference between the K_a values and dispersion of the inulin peaks from experimental and control animals

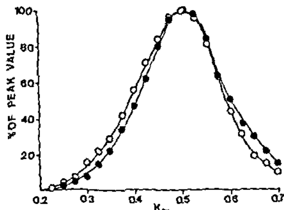


Fig 1 Gel filtration pattern of inulin in plasma from animals receiving an infusion of inulin (O—O) and of inulin dissolved in rabbit plasma from control animals to which no inulin has been administered (●—●) Each point denotes the mean of 2 expts

The gel filtration experiments as reported here show that the glomerular filterability of all fractions of inulin is similar, and therefore it may be concluded that even the most high molecular part of the polyfructoside is freely filterable through the glomerular membranes of the rabbit kidney The same conclusion has been reached by Mogensen (1968 *a*) in gel filtration studies of inulin in the human Furthermore, Mogensen (1968 *b*) and Arturson, Groth and Grotte (1971) have assessed the filterability characteristics of dextran of different molecular size in the glomerular membrane of adult humans These authors found that dextran of a molecular weight of more than 15 000 (corresponding to a Stoke—Einstein radius of appr 27 Å) was incompletely filtered According to Granath and Kvist (1967) commercial preparations of inulin contain substantial amounts of fructosides having a molecular weight of more than 15 000 However, it is apparent from the gel filtration results shown in this study that inulin has a more compact structure than dextran

The data of Table I, in conjunction with the gel filtration experiments as shown in Fig 2, indicate that the clearance of PEG 1500 and PEG-4000 like that of

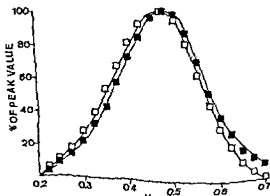


Fig 2 Gel filtration pattern of inulin in urine from animals receiving an infusion of inulin (□—□) and of inulin dissolved in rabbit urine from control animals to which no inulin has been administered (■—■) Each point denotes the mean of 2 expts

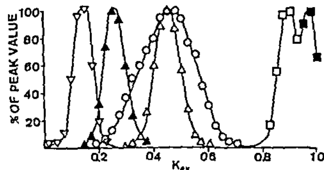


Fig. 3. Gel filtration analysis of aqueous solutions of inulin (○—○), PEG 1500 (△—△), PEG 4000 (▲—▲), PEG 6000 (▽—▽), raffinose (□—□) and fructose (■—■), containing 0.9 % NaCl.

inulin measures the glomerular filtration rate, while the passage of PEG 6000 across the glomerular membrane is restricted to some extent. The results found here for the rabbit are similar to those obtained by Shaffer, Critchfield and Carpenter (1948) on the renal excretion of polyethylene glycol in the dog. Besides, Hardwicke et al. (1968) investigating the gel filtration pattern of radioactive derivatives of dextran and polyvinylpyrrolidone in the rabbit have noted that the filterability characteristics of rabbit and man are similar. Thus, the glomeruli of rabbit, man and dog apparently are capable of filtering compounds of a greater molecular diameter than those of the rat (Berglund 1964, 1965, 1968).

Physico-Chemical Characteristics of Inulin and Polyethylene Glycol

A notable feature of the experiments reported above is that PEG 6000 is incompletely filtered by the glomeruli in spite of the fact that the molecular weight of this substance is similar to the average molecular weight of inulin which is approximately 6000 (Phelps 1965 and Westfall and Landis 1936). In order to compare the molecular dimensions of inulin and polyethylene glycol we have determined the gel filtration pattern of the two different types of substances (Fig. 3). Inulin, PEG 1500, PEG 4000 or PEG 6000 was dissolved in 0.9 % NaCl and chromatographed as described in the *Methods* section. It can be seen from Fig. 3 that the inulin peak is broader than those of PEG 1500, PEG 4000 and PEG 6000 indicating the heterogeneity of the polyfructoside preparation. However, inulin is eluted completely in advance of fructose and raffinose which were chromatographed in a few additional experiments in order to establish the elution volumes of mono- and oligofructosides. Therefore, it may be concluded that the inulin preparation does not contain free fructose or oligofructosides. Furthermore, the elution volumes of PEG 1500 and inulin are almost identical, whereas PEG 4000 is eluted at the leading edge of the inulin peak and PEG 6000 appears in advance of the polyfructoside.

Table II shows our ultrafiltration data on solutions of inulin and polyethylene glycol dissolved in 0.9 % NaCl. It is seen that inulin and PEG 1500 are filtered through cellophane membranes to about the same extent. Furthermore, the filterability of PEG 4000 and PEG 6000 is much lower than that found for PEG 1500

TABLE II Ultrafiltration of inulin and different preparations of polyethylene glycol through cellophane membranes

Substance	Original Concn mg/100 ml	Ultrafiltrate Concn mg/100 ml	Filterability %
Inulin	105	45	43
PEG 1500	58	28	48
PEG 4000	57	5	8
PEG-6000	53	0 2	0 4

It is apparent from the results obtained by gel filtration and ultrafiltration that the permeability properties of inulin and PEG-1500 *in vitro* are almost identical Further, the molecular dimensions of inulin are considerably smaller than those of PEG 6000, and it is reasonable to suppose that this circumstance accounts for the incomplete filterability of PEG-6000 In this connection it is of interest that determinations of the diffusion coefficients of inulin (Bunim, Smith and Smith 1937) and PEG 1500 (Rossi, Bianchi and Rossi 1958) are also similar These findings are in agreement with the idea that flexible polymers like polyethylene glycol have a very expanded structure (Tanford 1961) Another possible explanation for the disparity of the molecular size of polyethylene glycol and inulin would be that the former type of substance is hydrated to a higher extent than inulin However, the Stoke-Einstein radius of polyethylene glycol as determined from diffusion and viscosity measurements agrees well with the radius obtained from theoretical calculations, assuming no hydration of the polymer (Rossi, Bianchi and Rossi 1958) On the other hand the physico chemical data of Phelps (1965) indicate that the frictional coefficient of inulin is about 1 35 higher than that calculated for a spherical shape of the molecule which means that the polyfructoside is either extensively hydrated or has an elongated form. In any case the fact that the dimensions of different polymers of the same molecular weight may vary widely should make it possible in future experiments to decide whether the glomerular filterability of non electrolytes can be described in terms of sets of fixed pore size as suggested by Artursson *et al* (1971)

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Effect of Increased Afterload on the Inotropic State and Uptake of Free Fatty Acids in the Intact Dog Heart

By

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Abstract

Kjekshus, J and O D Mjos *Effect of increased afterload on the inotropic state and uptake of free fatty acids in the intact dog heart* Acta physiol scand 1972 84 415—427

In anesthetized dogs treated with atropine and pentolinium tartrate—a ganglionic blocking agent—the possibility was tested that release of myocardial catecholamines contributes to the [illegible]

[illegible] unchanged arterial FFA concentrations indicating adrenergic activation of the heart These responses were abolished following a similar rise in left ventricular systolic pressure (from 88 to 184 mm Hg) in reserpine treated dogs during ganglionic blockade Changes in left ventricular end diastolic pressure as well as myocardial oxygen consumption were similar in both groups The present study supports the conclusion that catecholamines are released from myocardial stores during augmented intraventricular pressure thereby explaining the increase in cardiac output However in the catecholamine-depleted heart cardiac output is maintained by the Frank Starling mechanism alone

Myocardial adaptation to increased afterload is dependent on two fundamental mechanisms One is described by the Frank—Starling law of the heart and relates increased force of contraction to increased diastolic volume Starling and Visscher 1927) The other is characterized by an increased force of contraction at a constant ventricular volume in diastole and involves an inotropic stimulation of the heart It is with the latter factor that the present study is concerned

Although it has been found in isolated hearts that an abrupt increase in the pressure developed by the ventricle is accompanied by an inotropic response (Anrep 1912 Rosenblueth *et al* 1959, Sarnoff *et al* 1960, Clancy *et al* 1968 Monroe *et al* 1968 Furnival *et al* 1970), its importance in the regulation of myocardial performance has been disputed (Sonnenblick and Downing 1963) and as yet there is no quantitative

assessment of its influence on myocardial performance in the intact animal Furthermore, the mechanism inducing this effect is not clear Recent evidence has shown that increased levels of intraventricular pressure are accompanied by a heightened rate of norepinephrine release into the coronary sinus blood in innervated and in denervated hearts (Monroe *et al* 1966, La Farge *et al* 1970) Accordingly, release of endogenous catecholamines has been suggested as a contributory mechanism for the myocardial adaptation to increased outflow resistance The present investigation was designed to test this hypothesis Variations in baroreceptor stimulation effect changes in catecholamine release that might overrule myocardial release by endogenous mechanisms It should also be emphasized that artefactual depletion of myocardial catecholamine stores is likely in the thoracotomized animal For these reasons, the experiments were performed during autonomic blockade (atropine and pentolinium tartrate) in intact animals Similarly treated reserpinized dogs were used for comparison The participation of an endogenous release of norepinephrine at increased intraventricular pressure levels was further tested with respect to the ability of catecholamines to enhance myocardial uptake of FFA from coronary circulation (Gusios and Felts 1963 Gold *et al* 1965, Cowley *et al* 1969)

Methods

Animal preparation Mongrel dogs weighing 15—25 kg were anesthetized with sodium pentobarbital (30 mg/kg b w i v) The left jugular vein was cannulated for coronary sinus catheterization and as a route for central venous infusions A short wide bore catheter (0 8 m i d) introduced through the left carotid artery was used for pressure recordings from the left ventricle (0—40 mm Hg and 0—200 mm Hg) (Statham P23Gb) The first derivative with respect to time (LV dP/dt) was monitored continuously Aortic pressure was measured through a catheter in the femoral artery (0—200 mm Hg) Cardiac output was determined by thermal dilution technique and myocardial blood flow by inert gas desaturation using hydrogen as indicator Hydrogen was measured polarographically in the coronary sinus as described previously by Aukland *et al* (1964) Myocardial oxygen consumption (MVO_2) was calculated as the product of coronary blood flow and coronary a v difference of oxygen and uptake of free fatty acids (FFA) as the product of coronary plasma flow and coronary a v difference of FFA O_2 was determined according to Aukland (1962) and FFA was assayed in plasma by the method of Dole (1956) as modified by Trout *et al* (1960)

Experimental procedure All dogs were given atropine (0 5 mg/kg b w) to avoid reflex bradycardia and sodium heparin (3 mg/kg b w) In each experiment control hemodynamic conditions were established When ventricular pressure heart rate coronary blood flow and cardiac output had become stable blood was sampled for determination of MVO_2 and FFA uptake Thereafter left ventricular systolic pressure was increased in one or two steps of
aorta (4 dogs) or by
step a minimum of
measurements were
whether pressure was
ffects of angiotensin
ese data have therefore been pooled

After discontinuing angiotensin infusion or deflating the balloon pressure returned to control levels One of two pharmaceutical agents—pentolinium tartrate (Ansolysen , 3—8 mg/kg) or propranolol (Inderal 0 50—0 75 mg/kg) was then given i v Stable hemodynamic conditions were established and MVO_2 and FFA uptake were measured before and during ang o-
systolic pressure to
e performed with

On two consecutive days prior to the experiments reserpine (0 25 mg/kg was given i p in 4 dogs. The effect of raising blood pressure on myocardial hemodynamics and on the metabolic state was examined before and after ganglionic blockade At the conclusion of the experiment the heart was rapidly excised and the content of catecholamines determined by the method of Anton and Sayre (1962)

Results

Myocardial function

a *Response to increased aortic blood pressure during ganglionic blockade* (Table I) Administration of pentolinium tartrate decreased left ventricular systolic pressure (LVSP), cardiac output (CO), dP/dt and heart rate (HR), and abolished the pressor response to carotid compression Stepwise increments in mean aortic blood pressure ($\overline{AP}$) caused increased CO and dP/dt in proportion to LVSP When LVSP was increased by an average of 94 mm Hg, CO and dP/dt increased by averages of 34 % ($p < 0.01$) and 50 % ($p < 0.005$), respectively The increase in CO occurred without changes in HR. Changes in CO and dp/dt are summarized in Fig 1 and 2, illustrating a direct relationship between changes in LVSP and CO and dP/dt, respectively during ganglionic blockade LVEDP increased by an average of 9 3 mm Hg (Fig 3)

Before administration of pentolinium tartrate, increments in $\overline{AP}$ resulted in depressed ventricular performance, in all animals studied, an increase in LVSP averaging 52 mm Hg was accompanied by a significant decrease in CO (on average 30 %) ($p < 0.001$) and dP/dt (on average 24 %) ($p < 0.01$), and by an average rise in LVEDP of 5 3 mm Hg HR was slightly reduced

b *Response to increased aortic blood pressure after reserpine treatment* (Table I and II) Administration of reserpine on two days prior to the experiment was sufficient to reduce myocardial catecholamines to less than 0 06 mg/g wet weight Since the experiments were performed within 48 hours after administration of reserpine, the direct negative inotropic effects of this agent could be avoided (Tanz and Marcus 1966, Roberts 1967) Reserpine administration resulted in reduced LVSP, dP/dt and HR, compared to normal dogs CO remained essentially unchanged (Table II)

Administration of pentolinium tartrate decreased LVSP CO and dP/dt slightly when compared with ganglionic blockade in non reserpinized dogs When LVSP was increased by an average of 96 mm Hg after ganglionic blockade CO dP/dt and HR were essentially unchanged, while LVEDP increased by an average of 8 2 mm Hg (Table I, Fig 1, 2, and 3)

When LVSP was augmented by an average of 47 mm Hg before ganglionic blockade dP/dt and HR were unchanged and CO fell, while LVEDP increased by an average of 3 2 mm Hg

c *Response to increased aortic blood pressure during β adrenergic receptor blockade* (Table I and II) β adrenergic blockade in 10 dogs attenuated but did not abolish the pressor and inotropic responses of carotid artery compression Consequently, reflex withdrawal of sympathetic tone during pressure loading could not

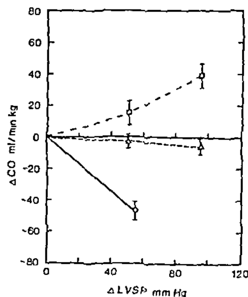


Fig 1

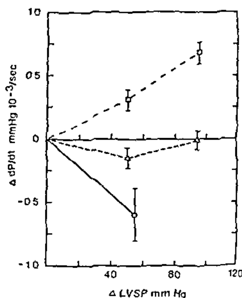


Fig 2

Fig 1 Changes in cardiac output (Δ CO) during raised left ventricular systolic pressure (Δ LVSP) in control dogs (○———○), after ganglionic blockade (□– – – –□) and after reserpinization + ganglionic blockade (△— — — –△) Mean values ± SE

Fig 2 Changes in maximal rate of rise of left ventricular pressure (Δ dP/dt) during raised left ventricular systolic pressure (Δ LVSP) in control dogs (○———○), after ganglionic blockade (□– – – –□) and after reserpinization + ganglionic blockade (△— — — — —△) Mean values ± SE

TABLE I Hemodynamic parameters before and during pressure loading in control state (C), after animals after ganglionic blockade (R+G) Mean ± SE

	LVSP mm Hg			LVEDP mm Hg			HR beats/min		
	I	II	III	I	II	III	I	II	III
C		137	192		0 7	6 0		143	138
n = 6		7	6		0 3	2 7		11	7
p		< 0 001			< 0 01			ns	
G	99	148	193	1 5	4 5	10 8	118	118	119
n = 6	5	5	7	0 3	1 1	2 0	7	8	9
p		< 0 001			< 0 005			ns	
G+B	98	152	194	1 8	5 8	11 2	109	107	113
n = 6	6	5	4	0 7	2 0	3 9	9	9	8
p		< 0 001			< 0 05			ns	
R+G	88	138	184	2 8	6 3	11 0	102	105	106
n = 4	6	9	11	0 5	1 6	1 8	5	5	6
p		< 0 005			< 0 025			ns	

I = unloaded condition II and III = during pressure loading n = number of dogs LVSP = pressure CO = cardiac output LVW = left ventricular work (CO × LVSP × 0 0136) MF = pressure ns = not significant (p > 0 05)

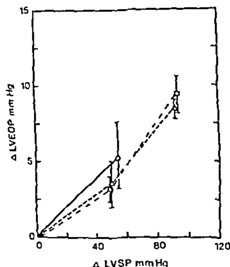


Fig 3

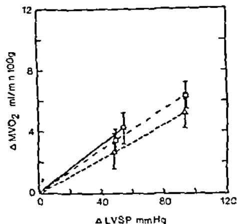


Fig 4

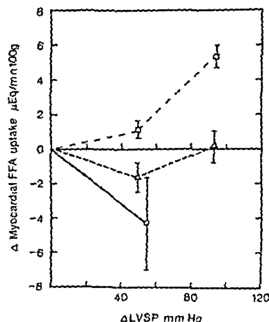
Fig 3 Changes in left ventricular end-diastolic pressure (ΔLVEDP) during raised left ventricular systolic pressure (ΔLVSP) in control dogs (○———○), after ganglionic blockade (□– – – –□) and after reserpinization + ganglionic blockade (△– – – – – –△) Mean values ± SE

Fig 4 Changes in myocardial oxygen consumption (MVO_2) during raised left ventricular systolic pressure (ΔLVSP) in control dogs (○———○) after ganglionic blockade (□– – – –□) and after reserpinization + ganglionic blockade (△– – – – – –△) Mean values ± SE

ganglionic blockade (G), after ganglionic blockade and propranolol (G+B) and in reserpinized

dP/dt mm Hg 10 ³/sec			CO ml/min kg			LVW kgm/min kg			MF ml min 100 g		
I	II	III	I	II	III	I	II	III	I	II	III
	2 50	1 90		150	104		0 28	0 28		113	124
	0 21	0 20		17	14		0 03	0 02		9	11
	< 0 01			< 0 001			ns			ns	
1 40	1 70	2 10	122	138	163	0 16	0 28	0 43	69	88	111
0 18	0 12	0 12	20	21	21	0 02	0 04	0 06	8	6	9
	< 0 005			< 0 01			0 005			0 001	
1 30	1 60	1 80	121	150	156	0 16	0 31	0 41	63	92	115
0 14	0 13	0 07	18	20	20	0 02	0 04	0 04	8	11	6
	< 0 02			< 0 01			< 0 005			< 0 005	
1 70	1 55	1 70	144	141	138	0 17	0 27	0 36	63	90	119
0 20	0.21	0 18	7	8	7	0 01	0 01	0 03	8	13	8
	ns			ns			< 0 005			< 0 005	

left ventricular systolic pressure HR — heart rate dP/dt = maximal rate of rise of left ventricular myocardial blood flow The probability values are given between initial and highest levels of blood



be eliminated Increments in LVSP from 128 to 174 mm Hg produced a reduction in CO almost similar to that seen in the control state, while dP/dt was unchanged, or tended to increase slightly (Table II) Administration of propranolol after ganglionic blockade had no effect on myocardial performance However following pressure rise CO and dP/dt rose The increases were only slightly less than observed during ganglionic blockade alone at a comparable increase in LVSP (Table I)

Myocardial metabolism

a *Response to increased aortic blood pressure during ganglionic blockade* (Table III) The general metabolic response to increased LVSP was indicated by a significant rise in MVO_2 averaging 4 2 ml/min 100 g (Fig 4) Although administration of pentolinium tartrate decreased MVO_2 in accordance with the hemodynamic changes increments in pressure comparable to those obtained in the control state also resulted in similar increases in MVO_2 Fig 4 shows MVO_2 for all dogs and illustrates that the increase in energy expenditure for a given rise in LVSP was essentially the same in all groups

Pentolinium tartrate lowered arterial concentration and uptake of FFA in accordance with its sympatholytic effect However when LVSP was increased by an average of 94 mm Hg during ganglionic blockade myocardial uptake of FFA rose markedly (221 %), while arterial concentrations of FFA remained unchanged In contrast when LVSP was increased by an average of 50 mm Hg before administration of penolinium tratrate arterial concentrations and myocardial uptake of FFA were reduced by an average of 40 % ($p < 0 05$) (Fig 5)

TABLE II Hemodynamic parameters before and during pressure loading in control state (C), after β adrenergic receptor blockade (B) and in reserpinized dogs (R) Mean ± SE

	LVSP mm Hg		LVEDP mm Hg		HR beats/min		dP/dt mm Hg 10^{-3}/sec		CO ml/min kg		LVW kgm min kg		MF ml/min 100 g	
	I	II	I	II	I	II	I	II	I	II	I	II	I	II
C	147	199	3.6	8.0	151	140	2.84	2.64	136	119	0.27	0.32	123	140
n = 10	9	6	0.8	2.2	9	8	0.26	0.29	17	16	0.02	0.03	10	12
p	< 0.001		< 0.01		ns		ns		ns		ns		ns	
B	128	174	6.0	10.1	116	121	1.41	1.73	88	80	0.15	0.19	85	120
n = 10	10	8	2.0	2.6	7	8	0.19	0.19	10	10	0.01	0.01	9	12
p	< 0.001		< 0.01		ns		ns		ns		ns		< 0.05	
R	109	156	3.8	7.0	102	114	1.85	1.85	153	137	0.23	0.30	84	102
n = 4	4	3	0.5	0.4	6	9	0.18	0.18	9	11	0.01	0.01	5	10
p	< 0.001		< 0.02		ns		ns		< 0.02		ns		ns	

Abbreviations as in Table I

TABLE III Metabolic parameters before and during pressure loading in control state (C), after ganglionic blockade (G), after ganglionic blockade and propranolol (G + B) and in reserpinized dogs after ganglionic blockade (R + G) Mean ± SE

	MVO_2 ml/min 100 g			MVO_2/HR µl/beat 100 g			Free fatty acids (FFA) a µEq/l			a-cs µEq/l			u µEq/min 100 g		
	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
C		12.2	16.4		86.8	119.2		697	421		172	112		10.5	6.3
n = 5		0.7	1.1		3.9	8.3		126	114		34	59		2.4	3.2
p		< 0.02			< 0.02			0.05			0.05			0.05	
G	8.0	11.4	14.3	67.7	98.5	122.7	424	349	415	70	75	143	2.9	4.0	9.3
n = 5	0.9	0.7	1.3	7.0	8.0	13.4	44	31	25	46	23	10	1.6	1.2	1.0
p		< 0.005			< 0.005			ns			0.05			0.001	
G + B*	6.3	10.7	13.5	58.6	100.8	121.7	345	309	369	48	47	64	2.0	3.1	5.0
n = 5	0.9	1.3	1.4	7.1	11.5	15.2									
p		< 0.005			< 0.005										
R + G	5.5	8.1	10.6	53.9	77.9	100.8	378	267	295	150	65	5	6.4	4.6	6.6
n = 4	0.4	2.0	2.0	6.3	20.7	21.3	25	37	52	32	21	12	2.5	1.8	1.2
p		< 0.05			< 0.05			ns			ns			ns	

I = unloaded condition II and III = during pressure loading n = number of dogs MVO_2/HR = stroke oxygen consumption a = arterial concentration, a-cs = arterio-coronary sinus difference u = myocardial uptake of free fatty acids The probability values are given between initial and highest levels of blood pressure *FFA values are means of two experiments ns = not significant (p > 0.05)

TABLE IV Metabolic parameters before and during pressure loading in control state (C), after β adrenergic receptor blockade (B) and in reserpinized dogs (R) Mean ± SE

	MVO_2		MVO_2/HR		Free fatty acids (FFA)					
					a		a cs		u	
	ml/min 100 g		µl/beat 100 g		µEq/l		µEq/l		µEq/min 100 g	
	I	II	I	II	I	II	I	II	I	II
C	12 5	16 0	82 8	114 3	775	558	171	118	11 4	9 7
n = 10	1 5	2 0	8 3	15 1	79	55	31	30	1 9	3 4
p	< 0 02		< 0 02		ns		ns		ns	
B	9 2	13 5	79 3	111 6	479	395	69	84	3 4	6 2
n = 10	1 6	2 1	8 8	15 8	45	57	18	11	0 9	0 7
p	< 0 02		< 0 02		ns		ns		< 0 05	
R	7 7	10 7	73 0	94 0	444	301	137	76	8 2	5 0
n = 4	0 7	1 4	4 0	12 1	63	49	22	29	1 7	2 1
p	< 0 05		ns		< 0 05		ns		ns	

Abbreviations as in Table III

b *Response to increased aortic blood pressure after reserpine treatment* (Table III and IV) Myocardial oxygen consumption was less in reserpinized than in non reserpinized dogs in accordance with the reduced myocardial performance When LVSP was raised similarly before and during ganglionic blockade the rise in MVO_2 was not significantly different from that in non reserpinized dogs

Arterial concentrations and myocardial uptake of FFA were lower than in non reserpinized animals and fell further with increased aortic blood pressure (Table IV)

Pressure loading after ganglionic blockade in reserpinized dogs resulted in virtually unchanged uptake of FFA in contrast to the marked increase in FFA uptake during increased afterload following ganglionic blockade in non reserpinized dogs (Table III) The absence of a positive contractile response to pressure loading in reserpinized animals thus coincides with lack of positive stimulation of myocardial FFA uptake

c *Response to increased aortic blood pressure during β adrenergic receptor blockade* (Table III and IV) Propranolol when administered to dogs before or after ganglionic blockade did not influence the relationship between increments in pressure and associated changes in MVO_2 A slightly increased (p 0 05) uptake of FFA was observed subsequent to increments in pressure in dogs treated with propranolol (Table IV) Administration of propranolol after ganglionic blockade produced a further slight decrease in arterial concentration and myocardial uptake of FFA however, increase in pressure induced a rise in myocardial uptake of FFA slightly less than the increase observed in afterloaded animals with ganglionic blockade alone (Table III)

Discussion

Previous experiments suggest that the response to increased aortic blood pressure includes a positive inotropic effect on the left ventricle (Anrep 1912 Sarnoff *et al* 1960 Monroe *et al* 1968) This is a delicate phenomenon easily masked by reflex changes in sympathetic tone induced by changes in baroreceptor activity Thus, the present study shows that increments in aortic pressure prior to the administration of ganglioplegics were followed either by no changes or by a depression of myocardial contractility, as evidenced by depressed dP/dt and cardiac output, whereas a positive inotropic effect was obtained in areflexic animals with intact myocardial catecholamine stores

Although the literature contains abundant evidence that cardiac output and dP/dt increase following the inotropic effect of catecholamines, it might be argued that the observed increase in dP/dt and cardiac output is not necessarily dependent on increased inotropic stimulation From the Frank-Starling relationship it follows that cardiac output tends to rise with increasing end diastolic pressure (Starling and Visscher 1927), and although dP/dt is determined by the velocity of the contractile elements it is also increased by factors not related to inotropic stimulation (Gilmore *et al* 1966 Sonnenblick *et al* 1969)

Although these relationships cannot be quantitated comparison between non reserpinized and reserpinized animals during ganglionic blockade provides quantitative information on the part played by myocardial catecholamines during increased ventricular pressure It is clear from the present study that similar increments in systolic and end diastolic pressures (and consequently end diastolic volume) during ganglionic blockade resulted in significantly increased dP/dt and CO in non reserpinized animals, while CO and dP/dt remained unchanged in catecholamine depleted dogs As long as the pre and post loading conditions are identical these parameters can be used for the assessment of differences in ventricular inotropic stimulating during increased pressure Since previous studies have demonstrated that the basic contractility is not influnced by reserpine (Tanz and Marcus 1966 Roberts 1967) as administered in the present study and that changes in nervous activity are excluded by atropinization and ganglionic blockade (autonomic blockade) the present observations strongly suggest an inotropic response to increased afterload mediated by catecholamines This implies that the increase in cardiac output over and above resting values is attributable to release of cardiac catecholamines while provision is made for maintenance of cardiac output by the Frank Starling mechanism alone

Results obtained with pressure loading in man Ross and Braunwald 1964 support the present finding Cardiac index increased in patients considered to have normal ventricular function, and was unchanged or decreased in patients with impaired ventricular function. It is well known that catecholamine stores are depleted in failing hearts (Chidsey *et al* 1963 1964) and in this respect therefore resemble reserpinized hearts

The finding that the positive inotropic response was abolished in hearts depleted

of catecholamines by reserpine is at variance with data referred to by Blinks and Koch Weser (1963) and experiments by Rosas *et al* (1965) However, although the latter investigators demonstrated that the positive inotropic response could be elicited in reserpinized animals it was greatly reduced It is possible that in their experiments the depletion of catecholamines might have been incomplete since ganglionic blockade resulted in a drop in arterial pressure of 40 mm Hg

Our observation that the magnitude of the hemodynamic response to increased aortic pressure was only slightly depressed following β receptor blockade is in accordance with studies by Clancy *et al* (1968), who found that the inotropic response to increased intraventricular pressure in isovolumetric preparations could be demonstrated after propanolol This is probably due to the fact that the effect of catecholamines released from the heart by sympathetic stimulation is only slightly depressed by propranolol, compared to the marked inhibition of catecholamines when administered exogenously as shown by Donald *et al* (1968)

It has been suggested that adrenergic mechanisms are involved in the myocardial uptake and utilization of FFA (Gusios and Felts 1963 Gold *et al* 1965 Glaviano and Masters 1967 Marchetti *et al* 1968) The finding that increased intraventricular pressure was parallelled by augmented myocardial FFA uptake in non reserpinized animals—whereas the uptake was virtually unchanged in reserpinized animals—supports the contention that increased intraventricular pressure is associated with release of catecholamines from the heart

Although arterial concentrations of FFA greatly influence uptake of FFA Evans *et al* 1963) this could not be the case in animals subjected to ganglionic blockade since arterial concentration of FFA remained essentially constant during pressure loading and differences in myocardial uptake of FFA were therefore not related to lipolysis outside the myocardium However pressure loading in animals with intact baroreceptor reflexes was followed by reduction in myocardial uptake of FFA Although this is in accordance with Glaviano and Masters (1967) and Marchetti *et al* (1968)—who concluded that the uptake of FFA was impaired by inhibition of specific adrenergic mechanisms—the diminished uptake of FFA in unblocked dogs is probably an indirect effect due to reduced arterial FFA concentrations (Akre and Mjos 1971)

Cowley *et al* (1969) Crass *et al* (1969) and Neely *et al* (1969) suggest that myocardial uptake and oxidation of FFA in the working heart are related to oxidative demand Efforts were made in the present experiments to increase the pressures to comparable levels The increase in MVO_2 was virtually the same in all groups, differences in oxidative demand were therefore minimal and could not account for the observed dissociation in FFA uptake between animals with intact myocardial adrenergic mechanisms and those depleted of catecholamines Indirect confirmation is thereby obtained of the role played by catecholamine release in myocardial adaptation to increased blood pressure

It has been postulated that increased sensitivity to angiotensin during ganglionic blockade is associated with a rise in peripheral vasoconstrictor response (Page and

Olmsted 1963, Gordon and Stephenson 1967) From the present study, however, it is suggested that the basis for the increased pressor response is an inotropic stimulation of the heart, rather than increased vasoconstrictor reponse This is in accordance with Zimmerman (1962), who found *reduced* vasopressor response to angiotensin in perfused hind-quarters of dogs after acute sympathectomy Furthermore the positive inotropic response is not a specific action of angiotensin, since a similar increase in CO and inotropic stimulation was obtained by inflating a balloon in the thoracic aorta of animals subjected to ganglionic blockade

It might be argued that the positive inotropic response to pressure loading after ganglionic blockade may be indirect due to release of catecholamines from the adrenal medulla (Feldberg and Lewis 1964, Peach *et al* 1966) or to sensitization of the heart to noradrenaline (Page and Olmsted 1963) However, if these mechanisms were operating a rise in heart rate would be expected, according to Spoerel and Gowdey (1956) They observed that noradrenaline raised heart rate markedly after ganglionic blockade, in contrast to unchanged or slightly decreased frequency when given before blockade Since, in the present study heart rate remained constant during the inotropic response to pressure loading after ganglionic blockade, it is unlikely that this mechanism is attributable to catecholamines released outside the heart

The results of the present investigation are relevant in the study of hypertension in man Young hypertensives have been shown to have a high cardiac output (Bello *et al* 1965) These hemodynamic changes might stem from the fact that resetting of baroreceptor activity takes place within a few days following augmentation of blood pressure (McCubbin 1958 Krieger 1970) Thus the baroreceptors no longer counteract the effect of high blood pressure on myocardial contractility, and consequently cardiac output will increase

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The Voluntary Regulation of Breathing in Man

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It has been shown that a distinct relationship exists between the electrical and mechanical activities in man s voluntary muscles (Lippold 1952 Bergstrom 1962) A corresponding relationship has been demonstrated in the respiratory muscles of the rat (Bergstrom and Karttula 1961) and in man (Viljanen 1967 Viljanen and Halttunen 1967)

The question rises how breathing in man can be subjectively controlled by the human brain that is by its macrostate (Bergstrom 1967) and how this control is comparable with the control of other voluntary muscles in man

In the present preliminary experiments, which are made with 5 healthy male subjects aged 20—29 the psychophysical method of category production (Stevens 1958) is used

The voluntary control of both inspiration and expiration is studied In the category production of inspiration numbers are named in an irregular order from 1—10 where 0 represents the functional residual capacity (FRC) and 10 the state of maximal inspiration The subject is instructed to inspire volumes of air whose subjective magnitudes are equally spaced among the 10 categories presented by the experimenter

The category production of expiration is studied in the same manner so that the subject always starts from the FRC state and inspires maximally which state is marked by 0 The subject then expires in random volumes between 1 and 10 Ten represents the maximal expiration

The results are compared with results obtained with force of handgrip which is registered with a dynamometer (Vigorimeter) using the same method of category production The category production in expiration gives an exponential function with exponent > 1 (Fig 1) which value was also obtained with the category production of force of handgrip (Fig 1) In the category production of inspiration the curve is not exponential but has somekind of S shape

It can be seen that expiration and force of handgrip give a similar psychophysical function which is not the case in inspiration This seems to indicate that inspiration is controlled in a way different from that in expiratory and hand muscles Expiratory muscles seem to resemble the ordinary voluntary muscles in their subjective control

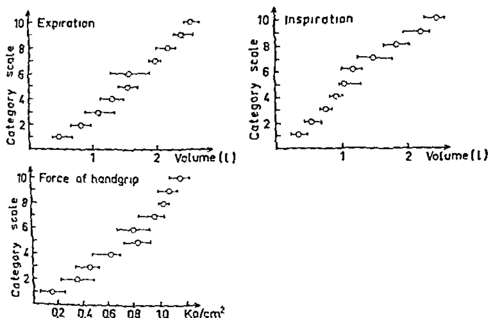


Fig 1 Motor psychophysical functions for handgrip, expiration and inspiration in one subject Each point (with range) represents the average motor response of *10* performances on the same category level

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the capsule with a glass rod These findings, together with the relative high threshold for electrical activation of the afferents, clearly indicate that these cells were linked with Ruffini endings in the joint capsule (Skoglund 1956)

During passive movements of the knee the activation range of these cells usually was the last 15—20° in the extreme of the movement (*cf* Burgess and Clarke 1969) This is not necessarily the normal activation range of the receptors as the tension in the capsule must have been profoundly changed by the dissection

First component units were activated by outward rotation of the foot and by flexion of the knee but were little sensitive to inflation of the capsule They could be activated by probing with a glass rod on and around the sesamoid bone in the tendon of the popliteus muscle or by pulling this tendon Therefore it is likely that these cells were linked with Golgi endings of the popliteus muscle, the afferents of which ' contaminated the posterior nerve of the knee joint (although muscle spindles can not be excluded in some cases *cf* Burgess and Clarke 1969) A few cells excited with short latency by volleys in the lowest threshold afferents in the medial nerve seemed to be connected with Golgi endings in the patellar ligaments

Impulses in afferents from joint receptors seem to be of great importance for man s ability to localize the position of a limb (Merton 1964) On the basis of the present results it is postulated that information from joint receptors also may play a role in cerebellar motor regulation

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Effect of Cooling on Synaptic Transmission through the Cuneate Nucleus

By

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Abstract

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… local cooling of the … the nucleus, … apidly if the … the afferent … gularly seen … it since fast … ased N wave, the transmission through the nucleus was always reduced This was tested in various ways measurement of the latency and probability of firing of single units, "killed end —recording from lemniscal fibres as a measure of number of discharging cuneate neurones and measurement of the size of thalamic and cortical field potential produced by impulses relayed through the … most likely due to a greatly increased synaptic … the afferent fibres as a con … se branches occurred During … nained strikingly high

When the nervous system is cooled the overall effect is one of depression However, numerous reports indicate that moderate degrees of cooling enhance various nervous processes This is said to apply particularly to synaptic transmission in various areas For example, Grundfest (1941), and Brooks, Koizumi and Malcolm (1955) reported that cooling of the spinal cord increased the monosynaptic reflex elicited by stimulation of muscle nerves The conclusion that cooling enhances synaptic transmission is however, contradicted by work on individual synapses

In both the giant synapse of the squid stellate ganglion and at the frog neuromuscular junction, cooling impairs the chances for synaptic transmission as indicated by an increased synaptic delay with an increased latency and a reduced probability of the postsynaptic discharge, gradually leading to a complete block of transmission (Bullock 1948, Katz and Miledi 1965) There was no transitory phase of improved synaptic transmission in either of these preparations

Moderate cooling does not only increase various extracellularly recorded trans synaptic responses but also compound action potentials of various peripheral nerves Thus, Lundberg (1948) found that cooling of peripheral nerves with myelinated and non myelinated fibres resulted in a large increase of the amplitude of the nerve ac tion potential, particularly in the C fibres Ritchie and Straub (1956) confirmed Lundberg s findings in unmyelinated nerves, and explained the phenomenon If the duration of the action potentials of individual fibres increases more than the con comitant slowing of conduction, the result will be a larger summed amplitude of the compound nerve action potential for purely geometrical reasons

Thus cooling produces two apparently opposite effects namely on the one hand increased nerve action potentials and ventral root monosynaptic reflex discharges versus on the other hand clearcut evidence of impaired synaptic transmission in isolated preparations In view of this paradox we wanted to reinvestigate the effect of cooling on transmission in a central synapse The cuneate nucleus was chosen for a number of reasons its superficial location allows for fast cooling and rewarming *the mechanism of synaptic transmission through this nucleus is well known* (Ther man 1941, Amassian and deVito 1957 Andersen Eccles Oshima and Schmidt 1964) A quantitative assessment of the number of discharging cuneate cells is available through killed end recording from the lemniscal afferents terminating in the ventrobasal complex of the thalamus much like the size of the ventral root mono synaptic reflex indicates the number of discharging motoneurones A further ad vantage in choosing the dorsal column nuclei for study is the well developed pre-synaptic inhibition which can be studied in some detail since the participating fibres are located very close to the dorsal surface of the brain stem (Andersen *et al* 1964 b Andersen Etholm and Gordon 1970)

Thus the main aim of the present investigation was to study whether or not moderate cooling had a transitory facilitatory effect on the transmission through the cuneate nucleus In subsequent papers the effect of cooling on inhibitory processes and the mechanism underlying the cooling effect on extracellular potentials are dealt with (Andersen Gjerstad and Pasztor 1972 Andersen *et al* 1972)

Methods

Adult rats were used anaesthetized with 30 mg sodium pentobarbital per kg given i.p After cannulation of the femoral vein and the trachea the dorsal column nuclei were exposed by removing part of the occipital bone and the dorsal part of the atlas and the atlanto-occipital membrane The superficial radial median and ulnar nerves were dissected in the right fore

tinum electrodes Silver ball electrodes
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l to record the field potentials and unit
the number of cuneate cells discharged
the ventrobasal complex with a coarse
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the killed ends of
Properly placed
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recording electrode correctly, accepting only responses of more than 0 2 mV In other experiments the focal potential of the ventro-basal complex was recorded with glass micropipettes. Surface cortical responses were recorded with silver ball electrodes located to the post-cruciate cortex just caudal and lateral to the dimple The temperature was measured with small thermocouples (diameter 0 5 mm length 0 8 mm) In the cortex the thermocouples were inserted into the cortical grey matter to a depth of 2 0 mm ensuring that the surface fluid did not reach the thermocouple itself To record the thalamic temperature, the thermocouple was inserted just lateral to the ventrobasal complex to a depth of 7 mm from the dorsal surface of the thalamus The temperature of the cuneate region was usually recorded from a depth of 2 mm in the left cuneate nucleus the right cuneate nucleus being used for recording of nerve cell responses The thermocouple potentials were recorded by using a fourth thermocouple in a thermos flask with crushed ice The potentials between the thermocouples in the brain and the reference thermocouple were led to a voltage to-frequency converter which fed an electronic counter The temperature was either read off directly from the counter or printed out on a paper strip All thermocouples were calibrated afterwards with water at 3 tempera

with suction a thick tube being necessary because of the greatly increased viscosity at this temperature In experiments with single unit recording local cooling was performed with a chamber of perspex with a silver bottom shaped after the surface of the dorsal column nuclei The chamber was irrigated with cool ethylene glycol or ethyl alcohol since an electrically conducting fluid introduced electrical disturbances in the recording circuit An eccentric pump circulated the cooling fluid through the chamber and a cooling coil in a thermos flask Two holes in the baseplate allowed the positioning of a thermocouple into the left cuneate nucleus

...ole to record unit activity in the

...poreal circulation of blood taken ... The cannula was connected to a ... within which the temperature of blood ... After being cooled or warmed in the thermos flask the

cooling of the whole animal by immersing it in ice water after initial anesthesia and washing of the skin with a detergent to abolish the insulating air trapped in the fur

Results

Effect of local cooling on cuneate field potentials Local cooling of the cuneate nuclear region produced dramatic changes in the potentials recorded from the surface or inside the nucleus in response to stimulation of a peripheral nerve (Fig 1) Depending upon the condition, the increase in amplitude could amount to several hundred per cent The effect consisted of an increase of all parts of the potentials in particular the initial diphasic wave which signals the first afferent fibre volley and the following negative (N) wave (Andersen *et al* 1964 a) The latter is due partly to the extracellular current associated with the excitatory postsynaptic potential and partly to the discharge of cuneate neurones The subsequent positive wav

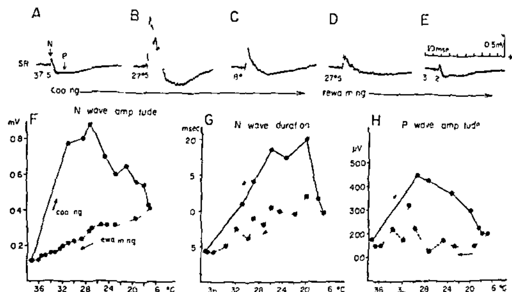


Fig 1 A—E Cuneate surface potentials in response to stimulation of the superficial radial nerve obtained during a period of cooling and subsequent rewarming Cuneate temperature as indicated The N and P waves are labelled in A F Amplitude of the N wave plotted against the local cuneate temperature during cooling (fully drawn line) and subsequent rewarming (broken line) Duration of N wave (G) and P wave size (H) as a function of the cuneate temperature The arrows indicate the sequence of observations during cooling and subsequent rewarming

(P wave) was also enhanced but much of the increase was counteracted by an increased duration of the N wave

The effect was dependent upon the temperature in a complex way With decreasing temperatures the N wave amplitude increased markedly (Fig 1 B F) In the experiment illustrated the maximal amplitude was obtained at around 27° C On continuing the cooling the responses started to decline (Fig 1 C F) The decline concerned the amplitude and later also the duration of the N wave (Fig 1 F G) On rewarming the potentials gradually declined but along a completely different curve than the one obtained during cooling In all figures the fully drawn lines indicate the cooling and the stippled line the rewarming sequence The hysteresis effect also applies to the behaviour of the duration of the N wave (Fig 1 G) Similarly, the P wave increased in amplitude during the first part of the cooling but was subsequently reduced to the control level This reduction is perhaps partly due to the masking effect of the enhanced N wave concealing the real extent of the P wave changes As expected the P wave duration also increased during cooling

The dramatic increase of cuneate potentials due to cooling particularly applied to the afferent fibre volley In Fig 2 A this volley is seen as a small deflection indicated by an arrow The fibre volley increased several hundred per cent by reduction of the cuneate temperature from 37° C to about 30° C (Fig 2 B E F) On further prolonged cooling the fibre volley was reduced in parallel with the N wave

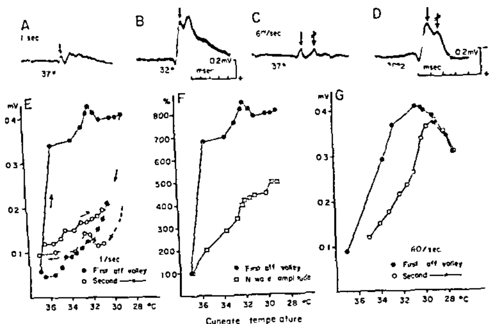


Fig 2 A C . . . 37° C and (B) during local cooling to 32° C

cuneate temperatures

On rewarming the afferent volley amplitude showed a clear hysteresis (Fig 2 E), but the potentials eventually attained their usual appearance Interestingly, the af ferent volley latency, measured to the peak of the initial positive part of the diphasic afferent volley deflection did not change appreciably during cooling In this it differed from the behaviour of the N wave the latency of which increased consider ably The amplitude of the fibre volley increased more than that of the N wave (Fig 2 F) By increasing the stimulation frequency to 160 s the N wave which is due to synaptic excitation was completely suppressed unmasking a second fibre volley (Fig 2 C, crossed arrow) This second deflection signalling the activity in thinner fibres was also augmented by cooling (Fig 2 D G)

The enhancement of the potentials by local cooling could be repeated over and over again with remarkably similar results On rewarming the potentials always re turned to the control values unless the cooling had been of severe degree and dura tion In such cases presumably irreversable ischaemic changes in the brain stem had been produced

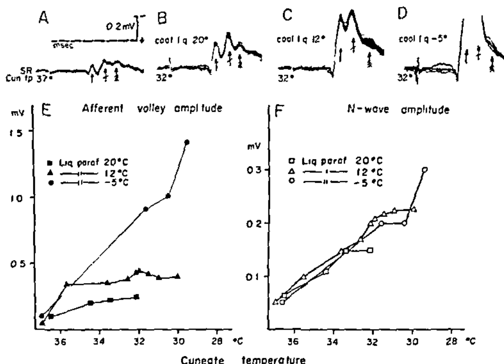


Fig 3 A—D Cuneate surface responses to a superficial radial volley The three arrows indicate the first three synchronous fibre volleys B, C and D were all taken during local cooling at a cuneate temperature of 32° C but with liquid paraffin of 20°, 12° and −5° C E Size of the first afferent volley plotted against local temperature during the three different runs reported above F Size of the N wave obtained in the same three experimental runs

The augmentation of the field potentials were obtained by all the cooling methods applied local perfusion either with cooled liquid paraffin or Ringer's fluid, cooling of the cuneate surface by way of a metal-floored cooling chamber, systemic cooling by immersion of the animal in ice and finally, extracorporeal circulation with re-introduction of cooled blood into the left carotid artery

Although the pattern of potential changes was the same irrespective of the cooling method, the degree of the change was dependent upon the speed with which the cooling was applied This parameter also determined at what temperature the increased potentials were maximal In the experiment illustrated in Fig 3, the surface of the cuneate nucleus was cooled with liquid paraffin of three different temperatures, with rewarming to full recovery between each run Fig 3 A shows the normal cuneate surface response to a superficial radial volley at a cuneate temperature of 37° C Three subsequent afferent fibre volleys are indicated by simple, crossed and feathered arrows The underlying N-wave was measured 1 msec after the last fibre volley B, C and D show similar responses to stimulation of the same nerve, recorded at a cuneate temperature of 32° C, but with cooling paraffin of 20°, 12° and −5° respectively The effect of cooling at different speeds on the amplitude of the first

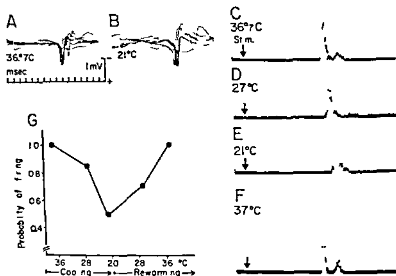


Fig 4 A Discharge of a single cuneate relay cell in response to 10/s stimulation of the ulnar nerve obtained at a temperature of 36 7° C B Same cell but at a cuneate temperature of 21° C C—F Sequence of poststimulus histograms taken from the same cell at the indicated cuneate temperatures Record length represents 15 13 ms G Probability of firing of the same unit at different local cuneate temperatures

afferent volley and of the N wave is seen in Fig 3 E and F The results could be taken to mean that the cuneate afferent volley potentials are not only dependent upon the tissue temperature, but also on the rate of change of this temperature However, it must be remembered that the temperature was monitored by a thermocouple at a tissue depth of 2 0 mm At the surface the temperature was probably lower, particularly when the coldest fluid was used

A similar rate dependent effect was observed by Pasztor and Kukorelli (1967) who found that the reduction of the cortical evoked response was dependent not only upon the degree but also on the rate of cooling

Effect of cooling on synaptic transmission in the cuneate nucleus

The greatly increased N wave led us to expect that the transmission across the synapses in the cuneate nucleus was increased by moderate degrees of cooling To our surprise this was not the case On the contrary the synaptic transmission to all relay cells was reduced This was born out by a series of different tests single unit recording with post stimulus histogram construction killed end recording of the medial lemniscus volley from the thalamic region recording of the thalamic N wave and recording of the cortical primary evoked potential all in response to stimulation of an appropriate fore limb nerve Fig 4 A shows about 10 superimposed traces with recording from a single cell from the cuneate nucleus in response to weak stimulation of the ulnar nerve This unit could be antidromically invaded from the me

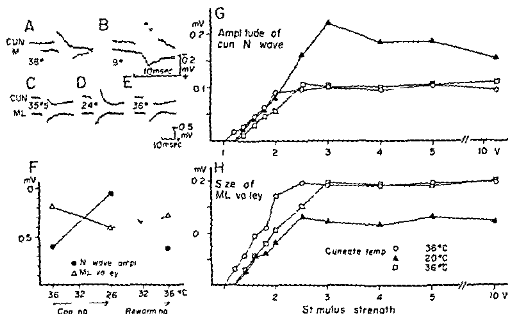
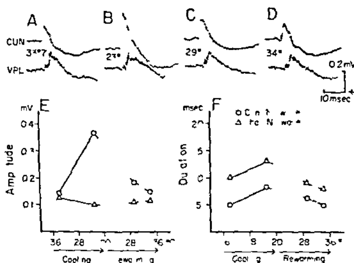


Fig 5 A and B Cuneate surface potentials (CUN upper traces) and medial lemniscus recordings (ML lower traces) at 38° and 19° C respectively following an ulnar nerve volley C D and E Average responses of 20 similar potentials obtained at the indicated local cuneate temperatures Size of the N wave and ML volley plotted in F G Amplitude of the cuneate N wave in response to stimulation of the ulnar nerve at the indicated stimulus strength The filled triangles were obtained during a cuneate temperature of 20° whereas the responses given by circles and rectangles were taken at 36° C before and after the cooling run respectively H Size of the ML volley during the same experimental situation

lemniscus At 36 7° C the unit fired relatively regularly with a latency of about 7 5 ms and occasionally discharged a second spike about 1 2 ms later Following cooling to 21° C (B) the second discharge hardly occurred and the latency and variability of the initial discharge were increased The apparent increase of amplitude was not a consistent feature of single unit recording during cooling It is probably due to a small movement of the electrode relative to the cell The latency effect is better seen in the poststimulus histograms in Fig 2 C—F where C shows the histogram recorded at 36 7° C before cooling (corresponds to A) Cooling to 27° C (D) increased the latency and duration of the first histogram deflection markedly and the second peak was nearly abolished At 21° C (E) the initial peak had a further increased latency and was much broader indicating a greater variability of the discharge time of the unit The second discharge had totally disappeared Following rewarming to 37° C (F), the post stimulus histogram attained a normal configuration with a remarkable constancy of the first discharge The overall probability of firing was decreased during the cooling as indicated by the graph in Fig 4 G The behaviour of this relay cell was typical of a large number of neurones and contrasted the behaviour of primary afferent fibres (see Fig 8)



Fg 6 A—D Surface potentials of the right cuneate nucleus (CU\) and f eld potent als of the left ventral posterolateral nucleus of thalamus (\PL) taken during cool ng of the cuneate reg on to the temperatures ind cated Ampl tude (E) and duration (F) of the two waves are plotted during cooling and subsequent rewarming S mbols gi en in F

Lemniscal recording The finding from single unit recording that the enhanced N wave of the surface record did not signify an increased synaptic transmission was verified by killed end recording of the medial lemniscal volley which gives a good estimate of the total amount of discharges emanating from the cuneate nucleus in the direction of VBC

In Fig 5 A the upper trace shows the surface cuneate potential (CU\) and the lower trace the medial lemniscus killed end response (ML) both taken at a normal temperature of 38° C In B the cuneate region was superfused with cool paraffin to reduce the local temperature to 19° C The thalamic temperature remained at 38° C The enhanced N wave was accompanied by a reduced killed end (ML) re ponse indicating a reduction of the number of cuneate cells dis harging towards the thalamus In order to measure the effect more exactly 20 potentials were aver aged at a cuneate temperature of 35 5° C (C) A reduction of the cuneate temperature to 24° C increased the averaged N wave whereas the medial lemniscal volley was clearly reduced (D) and had a longer latency Subsequent rewarming to a cuneate temperature of 36° C reestablished the control values of the potentials (E) These effects are plotted in F

In order to study whether the cooling affected synaptic transmi sion of impul es of a particular fibre type the N wave and the corresponding ML volley was at different strengths of the afferent volley at normal and reduced temperature Fig 5 G the relation between the stimulus strength and the the resu cuneate N wave is plotted before cooling (open circles) during 20° (filled triangles) and after rewarming (open squares) In H are

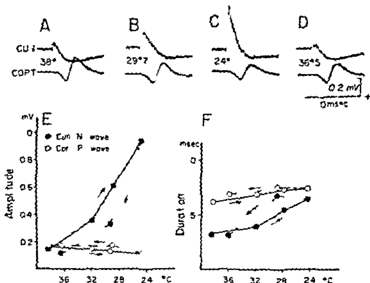


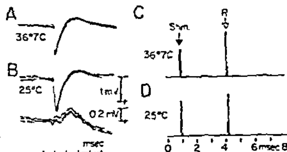
Fig 7 A—D Surface potentials of the right cuneate nucleus (CUN) and the left post cruciate cortical evoked potential (CORT) in response to ulnar nerve stimulation during cooling of the cuneate region to the temperatures indicated E Size of cuneate N wave and the positive cortical wave P wave (filled and open circles, respectively) during cooling (full lines) and subsequent rewarming (broken lines) F *Filled circles give duration of cuneate N wave* and open circles give duration of cortical P wave during the same cooling

obtained simultaneously from the medial lemniscus (same symbols) The cooling hardly affected the transmission of impulses produced by stimulation between one and two times threshold but from about two times threshold upwards the cooling effect was clear This indicates that the reduced ML volley is mainly due to blocking of synaptic transmission *of impulses in fibres of medium and small caliber*

Effect of local cuneate cooling on thalamic field potentials

Another way to estimate the degree to which the synaptic transmission in the cuneate nucleus is affected by local cooling is to study the size and form of the thalamic field potentials *produced by impulses from a peripheral nerve which are relayed* through the cuneate nucleus In Fig 6 the analogue signals along the top indicate the surface cuneate potential (CUN upper trace and the field potential recorded from the postero-lateral ventral nucleus of the thalamus VPL (lower trace) Cooling of the cuneate region to a temperature of 23° C without changing the thalamic temperature *of 37.5 C (Fig 6 B increased the afferent volley and the subsequent* N and P waves of the cuneate response However the corresponding VPL potential showed a small reduction In C and D are seen the return of the cuneate potential on rewarming again with little change in the thalamic field potential By measuring a large number of such individual records at different temperatures it is evident that the surface cuneate N wave increases *markedly (Fig 6 E)* whereas the thalamic field potential on the contrary showed a small reduction As expected the duration

Fig 8 A Response of a single afferent ulnar fibre at normal cuneate temperature 8 superimposed records. B Activity of the same fibre during reduced cuneate temperature The lower trace indicates the surface deflection showing coincidence with the primary afferent volley C and D Post stimulus histograms of 200 trials similar to those shown in A and B Stim. — stimulus artefact R — fibre response



of the thalamic N wave, which is produced by the lemniscal discharge, was also increased during cooling due to the delayed cuneate discharges through the lemniscus Another testimony towards the lack of an increased synaptic transmission during local cuneate cooling was derived from recording of single thalamic units In no case were increased number of discharges or decreased spike latencies observed On the contrary, the thalamic cells showed a small increase in latency and during prolonged cuneate cooling a reduced number of discharges

Size of primary evoked potential in sensori motor cortex A final test of the transmission across the cuneate synapses during local cooling came from recording of the evoked potential produced in the post-cruciate gyrus following stimulation of various peripheral nerves Concurrent with a clearcut increase of the cuneate N wave (Fig 7 C, CUN), there was a small reduction of size of both the positive and negative component of the primary somatosensory potential (Fig 7 C CORT The duration of this cortical potential was increased Following rewarming the potentials regained their control size and duration (Fig 7 E F)

Conduction in afferent fibres The reduced cuneate transmission could either be due to block of conduction in the afferent fibres or their terminal branches an increased synaptic delay, a reduced liberation of transmitter a decreased excitability of the postsynaptic membrane or a combination of some of these factors

The conduction along the stem of the afferent fibres was studied by impaling single myelinated axons of the dorsal columns before they turn down towards the deep-lying nucleus Impulse conduction in such thick myelinated fibres was resistant to cooling down to temperatures under 20° C In Fig 8 A and B are records taken from an ulnar afferent fibre in the dorsal column in the first cervical segment The identification of this unit as an afferent fibre rests upon the shallow depth of recording (0 5 mm) the short latency which coincided with the afferent volley deflection of the surface record (lower trace) by the high following frequency and finally on the extremely small variability of its discharge latency The latter point is brought out by the very narrow post stimulus histogram in C Local cooling to 25° C did not change the latency at all nor did it reduce the probability of firing

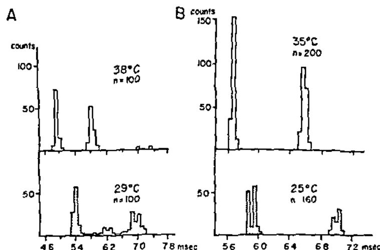


Fig 9 A Post stimulus histogram of a cuneate relay cell in response to stimulation of the ipsilateral ulnar nerve Open bars are at normal temperature of 38° C Hatched bars were taken during cooling to 29° C B Data from another cell The lower half show effect of cooling to 25° C

This behaviour stood in contrast to the terminal portions of the fibres which showed greater susceptibility to cooling Testing their excitability by the method of Wall (1958) the electrical threshold of the cuneate terminals inside the nucleus increased progressively as the temperature was reduced (Andersen Gjerstad and Pasztor 1972) The increase was particularly notable when the temperature was brought below 28° C This cold induced change indicated that the amount of charge which had to be removed per unit time to excite a fibre was increased with slowing of the conduction velocity as one of the consequences By comparing these data with similar measurements from the myelinated part of the fibres the slowing of conduction ought to be much greater for the thin terminal branches inside the nucleus than for the thicker parent fibres within the dorsal column itself (Andersen Gjerstad and Pasztor 1972) but the method does not allow a precise estimate to be made

Synaptic delay When measured as the time difference between the initial positive deflection of the afferent volley and the onset of the N wave in the surface records the synaptic delay increased with decreasing cuneate temperature The ML response also showed an increased latency and a diminished slope indicating an increased delay and reduction of the cuneate cell discharges during local cooling Finally by measuring the post stimulus histograms the synaptic delay was found to increase (Fig 9) Both the time to the onset and to the peak of the spike histograms increased as the cuneate temperature was reduced In Fig 9 A the latency of the first peak increased by 0 5 ms However judged by the latency of the antidromic nerve volley elicited by just suprathreshold intranuclear cuneate stimulation only 0 1 to 0 16 ms of this increase could be ascribed to an increased conduction time The

major part of the latency increase of the first peak for Fig 9 A is therefore likely to be due to an increased synaptic delay This interpretation is further supported by the greater variability of the discharge latency as indicated by the broadening of both histogram peaks obtained at 20° C particularly that of the second peak but with unchanged probability of discharge Other cells however, showed a slightly different pattern with a smaller latency increase and a hardly noticeable broadening of the histogram peaks in spite of severe cooling (Fig 9 B) In these cases, the major effect seems to be due to slowing of the conduction most likely in the terminal branches although accompanied by a reduction in the probability of discharge In conclusion the effect of cooling on the synaptic transmission in the cuneate nucleus seems to be due partly to a slowing of the conduction in the thin terminal fibres and partly to an increased synaptic delay probably caused by a slowing of the release of the transmitter

Discussion

Lack of enhanced synaptic transmission during cooling

The unchanged or slightly reduced synaptic transmission across the cuneate nucleus was born out by several methods all of which gave corresponding results The latency and number of discharged cuneate spikes the size of the "killed end medial lemniscal volley the size of the ventrobasal thalamic field potential as well as the latency and number of thalamic spikes and the size of the somatosensory evoked cortical potential all indicate that local cooling causes a reduced synaptic transmission through the cuneate nucleus

In view of the data from the cooled neuromuscular junction in frog (Katz and Miledi 1965) the most likely explanation for the cuneate results is that the normal nearly synchronous delivery of transmitter quanta is changed by the cooling to a more protracted release thereby increasing the latency and reducing the size of the EPSP and the probability for discharge The observed increase of the cuneate unit discharge latency is compatible with such an explanation However some cells showed changes that also can be ascribed to a slowing of conduction in the afferent fibres probably in their thin terminal branches Finally cooling also reduces the excitability of the postsynaptic membrane This effect is however most pronounced during relatively severe degrees of cooling (Andersen Gjerstad and Pasztor 1972 We are therefore left with a double explanation for the reduced synaptic transmission through the cuneate nucleus during cooling a delayed and protracted liberation of transmitter sometimes assisted by a slowing of impulse conduction in the afferent terminals The data does not allow an assessment of the relative importance of these two processes

The increased action and field potentials during cooling The paradoxical coexistence of greatly increased extracellular field potentials associated with excitatory processes and reduced synaptic transmission deserves some comments The problem will be dealt with in detail in another article Suffice it to say here that the observed

increase of compound potentials probably has a simple physical cause When the cuneate region is cooled locally the individual action or synaptic potentials have their duration increased much more than their time of onset Under such condition the degree of overlap between different individual potentials increases with a larger total height of the summed potentials Thus compound potentials like action potentials or extracellular EPSPs are larger although the number of participating neurones is the same or even slightly reduced (Ritchie and Straub 1956 Andersen *et al* 1972) This obviously also applies to the increased monosynaptic reflex The greatly increased discharge recorded from the ventral root when the spinal cord is cooled (Grundfest 1941 Brooks Koizumi and Malcolm 1955) is probably not due to an increased number of discharging motoneurones but to the summation effect explained above (Andersen *et al* 1972)

The hysteresis of the curves correlating the size of the afferent volley and N wave with local temperature may also have a simple explanation The initial rapid increase of these potentials was more pronounced for the fibre potentials than for the N wave (Fig 2) The reason may be that the thermocouple was located at a depth of 2 0 mm to record the nuclear temperature During the initial phase of cooling the surface temperature is certainly lower than the reading obtained by the deep thermocouple In the rewarming phase the reverse is probably true since the surface then was exposed to paraffin of 38°—40° C whereas the nuclear temperature recovered slowly for a period of several minutes In consequence the amplitudes observed during cooling appear too large If correction for the temperature gradient was possible the points should probably be shifted to the right reducing the apparent hysteresis effect However this can only be a part of the explanation The definite fall of the amplitude during the last part of the cooling (Fig 1) which was most pronounced for the N wave probably means that the number of participating neurones was reduced This would occur after some time when the surface cooling affected the nuclear temperature sufficiently At least two possibilities exist to explain the reduction One is that the cooling after a transitory phase of increased synchrony as described above has reduced the conduction velocity of the afferent fibres so much that a reduced synchronization of individual EPSPs and their corresponding discharges takes place Alternatively the cooling may have blocked conduction altogether in some fibres giving a smaller N wave as a result Of these two the latter explanation seems the most plausible

An additional factor which may contribute to the hysteresis of the N wave amplitude is the altered duration of the cuneate cell discharges which contribute to the N wave During surface cooling current will leave the cool superficial parts of the cuneate region to enter the cell bodies and initial parts of the axons which is at a higher temperature At a later stage the cell bodies will also be cooled with a broader action potential and an altered density of the extracellular current associated with the discharge which produces the field potentials

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Effects of Cooling on Inhibitory Processes in the Cuneate Nucleus

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Abstract

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Inhibitory mechanisms in the dorsal column nuclei have been studied during local cooling. The inhibition of transmission of a cutaneous volley across the cuneate nucleus caused by a preceding volley to the same or a neighbouring cutaneous nerve was prolonged and slightly increased by a moderate (25—30° C) reduction of the cuneate temperature. The excitability of presynaptic fibres and of the postsynaptic cell membrane both decreased progressively with decreasing temperature. Presynaptic depolarization increased during moderate cooling (25—30° C) but disappeared when the temperature was reduced to about 20° C.

One type of interneurone in the cuneate nucleus showed a progressive disruption of the normal pattern of firing with a gradual increase in the initial latency. Another type showed an increase of the rate and duration of the discharges to a single afferent volley on cooling. The latter type was susceptible to stimulation at 10 per s or higher. The behaviour of the latter interneurones matched the increased and prolonged presynaptic depolarization. These cells may be interneurones in a presynaptic inhibitory pathway. The prolonged and moderately increased inhibition caused by local cooling is probably due to activation of such cells causing increased presynaptic depolarization.

In the preceding paper (Andersen, Gjerstad and Pasztor 1972) we reported that the increased field and nerve action potentials seen during cooling of the dorsal column nuclei is not associated with an enhanced synaptic transmission. On the contrary, local cooling resulted in a reduced transmission as indicated by recording the cuneate cell discharges and their postsynaptic effects in the ventrobasal thalamus and postcruciate cortex. These effects can either be explained by a reduced synaptic transmission *per se* or by an increase of inhibitory processes, or a mixture of the two.

The present study was undertaken to investigate whether increased inhibition can give an explanation for the reduced transsynaptic effect, and if so, whether both presynaptic and postsynaptic inhibition were involved.

Methods

The methods were similar to those reported in the previous article (Andersen, Gjerstad and Pasztor 1972). The excitability of the primary afferent fibres were tested according to the method of Wall (1958), either by stimulation through a tungsten microelectrode in the nucleus

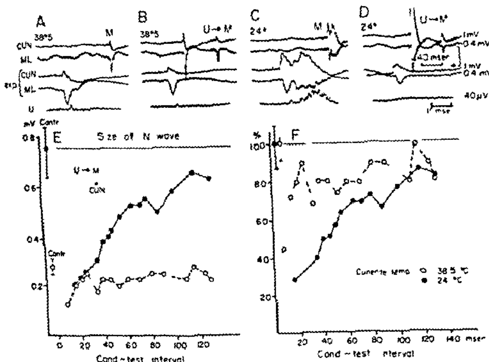


Fig 1 In A—D the upper two traces give the right cuneate surface response (CUN) and the left medial lemniscus response (ML) to stimulation of the right median nerve The next two traces show the same responses on an expanded sweep (exp) The fifth trace gives responses of the right ulnar nerve (U) on the same expanded sweep In B a stimulus was delivered to an ulnar nerve 60 ms earlier A and B were taken at 38 5° C cuneate temperature C and D are similar to A and B but obtained at a cuneate temperature of 24° C E Open circles give the size of cuneate N wave to stimulation of median nerve stimulation conditioned by an ulnar shock The conditioning test interval is shown along the abscissa The control level is indicated by the dotted line The filled circles were similarly obtained but at a cuneate temperature of 24° C The control value during the cool condition is indicated by the fully drawn line F The same data as in E replotted in per cent of the control values

or through a small platinum ball electrode resting upon the surface of the nucleus. The excitability of the postsynaptic membrane was estimated by stimulating the cells through an inserted tungsten microelectrode and recording of the killed end medial lemniscal (ML) volley by a coarse silver wire electrode insulated except for the squarely cut tip which was located within the medial lemniscus where it enters the thalamus at its ventral border For an estimation of the interneuronal activity which may be associated with presynaptic inhibitory processes records were made from cuneate neurones identified as interneurones by following the criteria of Andersen *et al* (1964 b)

Results

Effect of cooling on the inhibition in the cuneate nucleus In addition to excite a number of neurones an afferent volley to the cuneate and gracile nuclei evokes a profound inhibition as indicated by a reduction of the response to a subsequent test volley (Marshall 1941 Gordon and Jukes 1964 a b Andersen *et al* 1964) The in

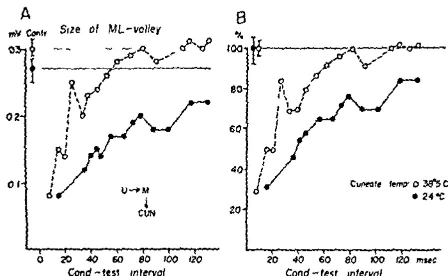


Fig 2 A Size of the medial lemniscus volley to a median nerve stimulus conditioned by a preceding ulnar nerve volley The conditioning test interval is indicated along the abscissa Results obtained during a cuneate temperature of 38 5° C indicated by open circles, those at 24° C by filled circles The control levels are indicated by dotted and full lines respectively B The same data plotted as per cent of the control values

hibition is both of a presynaptic and postsynaptic nature (Andersen *et al* 1964, Andersen Etholm and Gordon 1970) The most satisfactory method for testing the total effect of both components is to assess the reduction of the discharge of all cuneate relay neurones This can be done by measuring the size of the medial lemniscal (ML) volley when recorded with the killed end technique Another indicator of inhibition is the reduction of the N-wave which signals both the EPSPs produced in cuneate neurones as well as their spike discharges

Fig 1 A shows the cuneate surface record (CUN) with the N-wave and P wave (upper trace) and the contralateral medial lemniscal volley (ML) recorded with the "killed end" technique (second trace) in response to stimulation of the median nerve The third and fourth traces show the same two responses with an expanded sweep The fifth and last trace is a recording from the ulnar nerve with the same time base When a stimulus to the ulnar nerve preceded the test response by 60 ms, the test N-wave showed a moderate reduction and the P-wave a stronger one (Fig 1 B) There was also a small diminution of the ML volley in accord with the moderate inhibitory effects which are expected at this relatively long interval between the conditioning and the test stimuli On cooling to 24 C however there was a much more pronounced effect First the test response (Fig 1 C) now showed a larger N wave followed by a new negative deflection When the same conditioning stimulus as used in B, preceded this test response the relative diminution of the N-wave was more marked than normally (Fig 1 D) In particular, the last part of this wave was totally abolished, as were also the simultaneously occurring late deflection in the ML

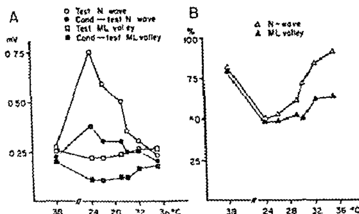


Fig 3 A Open circles and squares indicate the size of the cuneate N wave and the ML deflection respectively in response to stimulation of the median nerve at various cuneate temperatures The filled circles and squares indicate the size of the same responses when they were conditioned by an ulnar nerve volley elicited 40 ms earlier B The same data plotted as per cent of the control values at the appropriate temperatures

record and the antidromically conducted impulses in the ulnar nerve (fourth and fifth traces in C and D) All these three potentials are manifestations of the dorsal column reflex (Andersen *et al* 1964 a) This reflex which corresponds to the dorsal root reflex of the spinal cord (Barron and Matthews 1938 Toennies 1938) is caused by the depolarization of primary afferent fibres terminating in the dorsal column nuclei In a proportion of these fibres the depolarization will reach the threshold for impulse discharge The impulses are conducted both antidromically into the appropriate peripheral nerve (C lower trace) and orthodromically to excite cuneate neurones with the corresponding N wave and discharge along the lemniscal fibres (C third and fourth traces) The large increase of all these responses during cooling is similar to the well known augmenting effect of reduced temperature on the dorsal rooth reflex (Barron and Matthews 1938 Toennies 1939 By increasing the rate of stimulation from 1 to 10 or 20/s the cold induced increased dorsal column reflex in the nerve as well as the late component of the N wave and the late component of the ML record all disappeared suggesting that they were linked together and corresponded to the same frequency sensitive process

The graph in Fig 1 E gives the size of the test N wave expressed as a function of the conditioning test interval at 38° C (open circles and 24 C filled circles In F the same data are plotted as per cent of the control values using the same symbols as in E The reduction of the N wave was more marked at the reduced temperature, but the duration of the depression was not different from the condition during normal temperature

More direct evidence for the effect of cooling on the magnitude of the inhibition was derived from the size of the medial lemniscal (ML volley In Fig 2 A the size

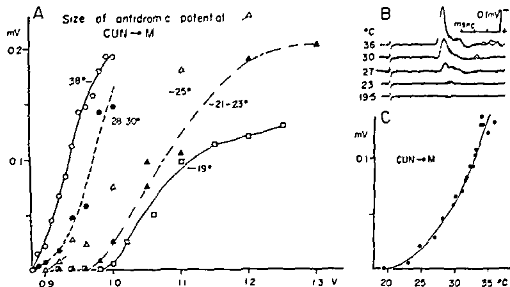


Fig 4 Excitability of primary afferent terminals during cooling A Size of the antidromic nerve action potential recorded from the median nerve in response to increasing stimuli delivered to the ipsilateral cuneate nucleus through a tungsten microelectrode The different graphs were taken at the indicated cuneate temperatures B Antidromic responses of median nerve to stimulation of the ipsilateral cuneate nucleus with 0 96 V taken at the indicated temperatures during a rewarming period Series should be read upwards C Size of the antidromic potential of median nerve to cuneate stimulation at the indicated temperatures during a rewarming period

of the ML volley produced by a stimulus to the contralateral median nerve (Fig 1 A ML second and fourth traces) is plotted against the interval between a preceding ulnar nerve stimulus and the test stimulus The control mean value and range of observations are given to the left The values obtained during reduced cuneate temperature (24 C filled circles) were markedly lower than those found at normal temperature (38 5 C open circles) indicating a more pronounced inhibitory effect during cooling In Fig 2 B the same data are plotted as percentage of the control values This graph indicates that the maximal degree of inhibition are similar at the two temperatures tested but that the inhibition during cooling is more pronounced at longer conditioning test intervals indicating that the inhibitory process lasts longer during cooling This effect comes in addition to the cold induced reduction of the number of cuneate cell impulses discharged along the medial lemniscus as indicated by the diminished ML control response during cooling (Fig 1 C 2 A)

In order to test whether the degree of inhibition at intermediate conditioning test intervals is a linear function of the cuneate temperature the effect of an ulnar volley on the response to a median nerve stimulus delivered 40 ms later was measured at different temperatures of the cuneate region In Fig 3 A the size of the unconditioned test N-wave is given as open circles The typical amplitude increase with reduced temperature is evident An ulnar volley, delivered 40 ms earlier, reduced the

test N wave at all temperatures (filled circles) The diminution was relatively greater at the lowest temperatures The corresponding data for the ML volley is plotted with open and filled squares Cooling reduced the unconditioned test ML response moderately (open squares) The inhibitory effect of the preceding ulnar volley was relatively stronger at the lower temperatures adding to the primary depressive effect of the cooling on the transmission. The relative effect on the N wave and the ML volley is better seen in Fig 3 B where the size of the N wave and ML volley are plotted as percentages of the unconditioned test responses at the indicated temperatures At temperatures below 20° C the size of both potentials were too small to allow a proper study

Effect of local cooling on the excitability of the primary afferent fibres The terminal portion of the primary afferent fibres is a possible location for the action of reduced temperature If one effect of cooling of the dorsal column nuclei is to reduce the membrane conductivity of the presynaptic fibres and hence of the conduction velocity *their excitability should decrease This can be detected by the test designed* by Wall (1958) Such studies are also necessary to evaluate the excitability tests for presynaptic depolarization

The excitability of the terminal fibres in the cuneate nucleus was measured by delivering a series of increasing stimuli through a small (2 μ) tungsten microelectrode located in the nucleus and measuring the resulting antidromic action potential in three peripheral nerves (superficial radial, median or ulnar) which were dissected in the fore limb of the animal and kept at a temperature between 33 and 37° C At a normal cuneate temperature the input-output curve for the median nerve fibre terminals was quite steep (Fig 4 A) When the cuneate region was cooled the threshold of the fibre terminals increased The slope of the input-output curve also decreased indicating that the reduction in excitability affected all fibre types in the sample During cuneate cooling the maximal antidromic potential in the median nerve was similar to the control value down to cuneate temperatures of 21° C indicating that it was possible to activate all fibres if the stimulating current was raised sufficiently Below this temperature level some fibres were blocked as signalled by a smaller maximal amplitude of the antidromic nerve action potential

Since only the cuneate region (including the first two cervical segments) were cooled the observed effects are largely attributable to the terminal part of the fibres In the experiment illustrated in Fig 4 B C the stimulus was kept constant at a strength that was subthreshold at a cuneate temperature of 19 5 C and the dorsal column nuclear region was allowed to rewarm spontaneously With increasing temperature the action potential gradually returned to the control value During the recovery there was a steady decrease of the latency indicating that the fibres were suffering reduced conduction velocity during cooling The thick β fibres recovered before the thinner δ fibres as judged from their latencies The deflections seen in Fig 4 B are true antidromic potentials except for the very last part of the record taken at 36° C which is due to the dorsal column reflex starting about 3 msec after the initial antidromic nerve action potential

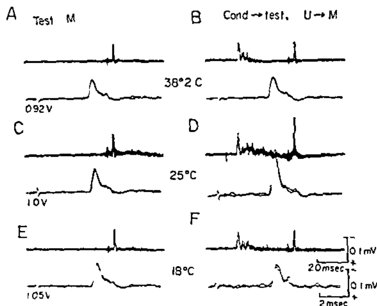


Fig 5 Presynaptic depolarization during cooling Each pair of traces shows the response recorded from the median nerve on a normal (upper) and expanded (lower) trace The left column (A, C, E) shows the antidromic median action potential in response to stimulation of ipsilateral cuneate nucleus with the strength indicated under each pair at cuneate temperatures of 38 2° C, 25° and 18° C respectively The right hand column shows the effect of a preceding ulnar volley, delivered 40 ms before the test shock

Effect of cooling upon presynaptic depolarization The increased duration of inhibition during cooling could be due to an increased presynaptic depolarization, which has been associated with presynaptic inhibition of transmission of impulses through the dorsal column nuclei (Andersen *et al* 1964 a c) Although it is well known that a reduced temperature increases the processes associated with presynaptic depolarization (Barron and Matthews 1938, Eccles, Magni and Willis 1962), it is not known whether the effect matches the increased inhibition of transmission during cooling We used the method of Wall (1958) to test the temperature dependence of the cuneate fibre depolarization As expected cooling enhanced the presynaptic depolarization in the cuneate nucleus There was an optimal temperature for the effect, below which the depolarization was gradually diminished In Fig 5 A the upper trace is a recording from the median nerve in response to a stimulus of 0 92 V delivered to the ipsilateral cuneate nucleus through a tungsten microelectrode The lower trace shows the same response on an expanded sweep The temperature of the cuneate region was 38 2 ° In Fig 5 B, this test response was preceded by a stimulus to the ulnar nerve, three times the threshold for the nerve action potential The ulnar volley created a dorsal column reflex in the median nerve as seen by the initial deflections in the upper trace, and also caused a slight increase of the antidromic

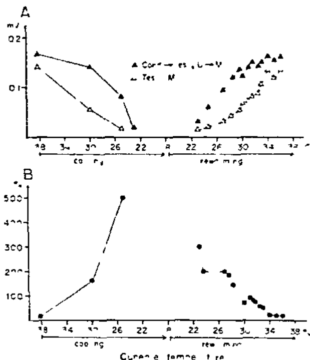


Fig 6 Relative effects on terminal excitability and presynaptic depolarization during cooling A Open triangles give size of the antidromic median nerve potential to stimulation of the ipsilateral cuneate region at the indicated temperatures The filled triangles give the increased antidromic potential caused by a preceding ulnar nerve stimulus delivered 40 ms earlier during cooling and subsequent rewarming B Same data as in A The percentage increase of the antidromic median nerve potential plotted as a function of the local cuneate temperature

nerve action potential delivered 40 ms later This increase, signalling presynaptic depolarization of the primary afferent fibres was moderate because of the relatively long interval between the two stimuli The records shown in C and D are similar to A and B, but were obtained at a cuneate temperature of 25° C The threshold for the antidromic action potential was increased, so the stimulus had to be raised to 1 00 V in order to elicit a response with an amplitude similar to that in A At this temperature, the dorsal column reflex following the antidromic volley by about 3 msec, was increased (C, upper trace) The dorsal column reflex elicited by the ulnar volley (Fig 5 D), was also increased and so was the antidromic test action potential in the median nerve, indicating an increased presynaptic depolarization The greatly prolonged dorsal column reflex shows the necessity for using a long conditioning test interval to avoid occlusion with the test volley The fact that the degree and duration of the inhibition of transmission through the cuneate nucleus was increased during the same experimental condition (Fig 2 B) suggests that the increased presynaptic depolarization could be associated with an augmented presynaptic inhibition as well Further cooling to 18° C again raised the threshold requiring adjustment to elicit a test response as in A At this temperature the dorsal column reflex was less well developed Similarly the presynaptic depolarization as signalled by the size of the antidromic median volley was not increased above the control level at this conditioning test interval (Fig 5 F At shorter intervals some

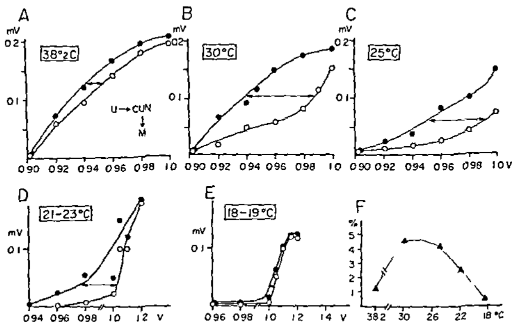


Fig 7 Temperature dependence of presynaptic depolarization A—E Open circles give the size of the antidromic median nerve action potential in response to stimulation of the ipsilateral cuneate region with the indicated strengths through a tungsten microelectrode The filled circles show the increased responses produced by a conditioning ulnar volley, delivered 40 ms earlier Each graph obtained at a cuneate temperature indicated in the boxes F Percentage increase of the presynaptic terminal excitability against the local cuneate temperature Method of Eccles, Magni and Willis (1962)

presynaptic depolarization remained but at a temperature between 18 and 15° C all traces of dorsal column reflexes and of increased antidromic nerve action potentials disappeared in all experiments

The complex changes of the properties of the primary afferent fibres caused by cooling is further illustrated in Fig 6 In A the open triangles give the size of the antidromic nerve action potential in the median nerve in response to a standard test stimulus delivered to the cuneate nucleus at the indicated temperatures The reduction of the response during cooling indicates the reduced excitability of the terminal parts of the median nerve fibres The filled triangles show how an ulnar nerve stimulus, delivered 40 ms earlier increased the test response The vertical distance between the two curves is an index of the increase of the fibre excitability produced by the conditioning volley or in other words of presynaptic depolarization This process was most pronounced between 30° and 25° C both during cooling and rewarming This can also be seen in Fig 6 B where the relative amplitude increase has been plotted as a percentage of the test responses at the different temperatures The lack of data under 23° C in this experiment is due to the abolition of the test response at this temperature

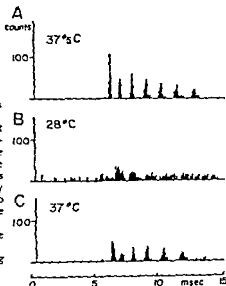


Fig 8 Effect of cooling upon a presumed excitatory interneurone Poststimulus histogram of a cuneate neurone that was not antidromically excited from the contralateral medial lemniscus, but which gave repetitive responses to all three forelimb nerves tested A was obtained at a cuneate temperature of 37 5° C and shows the remarkable regularity of the discharge This regularity was hardly changed by raising the stimulus frequency from 1 to 10/s. B was taken during cooling to 28° C and shows a much less regular discharge pattern although the rhythmicity still can be seen The intervals between subsequent peaks in the histogram are increased, and the spontaneous activity has increased C Control after rewarming to 37° C

A more detailed estimation of the degree of presynaptic depolarization at different cuneate temperatures is given by Fig 7 The experimental conditions were similar to those shown in Fig 5 and 6 Recording from the median nerve, a series of increasing test stimuli were delivered to the cuneate nucleus (A, open circles) Later each test response was conditioned by a standard ulnar volley delivered 40 ms earlier (filled circles) Following the method of Eccles Magni and Willis (1962) the degree of the presynaptic depolarization was estimated by comparing the size of the conditioned test response with the increase of the stimulus strength necessary to produce an unconditioned test response of the same size These values are indicated by the length of the horizontal arrows joining the two curves of each graph Again the largest degree of presynaptic depolarization was observed at temperatures of 30 and 25° C (Fig 7 B C) At lower temperatures the effect was considerably smaller (Fig 7 D, E) The degree of primary afferent depolarization is plotted against cuneate temperature in Fig 7 F The percentage increase was smaller in this experiment than is usual when the test stimuli are delivered through microelectrodes and at normal temperature This discrepancy is explained partly by the smaller ratio between threshold and maximal stimuli using a tungsten microelectrode of relatively low impedance as stimulating electrode in stead of glass pipettes Furthermore in order to avoid occlusion with the prolonged dorsal column reflex, the conditioning test interval had to be much longer than the optimal difference of about 15 ms (Andersen Eccles Schmidt and Yokota 1964 a)

Effect of cooling on postsynaptic excitability and inhibition We modified the method devised by Renshaw (1940) for spinal cord work for testing of the excitability of the cuneate postsynaptic elements On local cooling the response to micro-

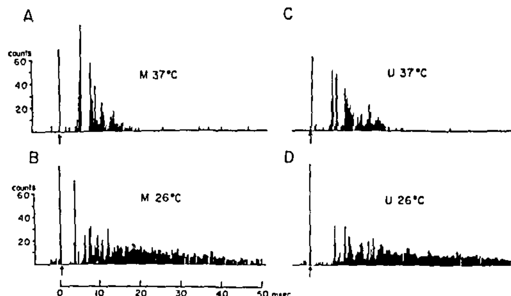


Fig. 9. Effect of cooling on a presumed presynaptic inhibitory interneurone. A and B show post-stimulus histograms of an interneurone of the cuneate nucleus obtained at cuneate temperatures of 37 and 26° C in response to median nerve volleys. C and D show the similar responses of the same cell to ulnar nerve stimulation.

electrode stimulation in the cuneate nucleus gave a reduction of the direct volley recorded from the medial lemniscus with the killed end technique. A preceding afferent volley in one of the ipsilateral forelimb nerves produced a reduction of the response to a standard test stimulus indicative of postsynaptic inhibition (Andersen *et al.* 1964). However, cooling to 25° C had little effect on the relative reduction of the test response, although the absolute size of the latter diminished. The lack of an effect refers to both the size and time course of the inhibitory curve.

In conclusion, local cuneate cooling caused a definite reduction of the excitability of the postsynaptic membranes much as Brooks, Koizumi and Malcolm (1955) found for motoneurones. However, postsynaptic inhibition was not significantly changed during the same procedure.

Effect of cooling on interneurones. Following the criteria of Andersen *et al.* (1964 c), we define interneurones in the cuneate nucleus as cells that 1) are not antidromically invaded from the medial lemniscus but 2) are activated from more than one, and often all three of the forelimb nerves we dissected and 3) often respond to a single stimulus with a long repetitive discharge.

Of the cuneate neurones identified as interneurones and kept sufficiently long, about half showed a pure depressive reaction to lowering of the local temperature as indicated by an increased latency, a reduced probability of firing and disruption of their often remarkably regular pattern of high frequency discharge (Fig. 8 A–B). Quite often the spontaneous activity increased during cooling as seen by the

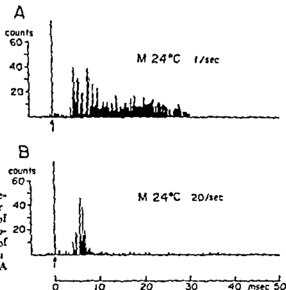


Fig 10 Frequency sensitivity of presumed presynaptic inhibitory interneurone A Post-stimulus histogram of the same interneurone as in Fig 9 obtained at a cuneate temperature of 24° C in response to 1 per sec stimulation of the median nerve B As A but stimulation at 20 per s

occurence of discharge between 0 and 6 msec latency (Fig 8 B) The effect was reversable (Fig 8 C)

The other type of interneurone we found reacted quite differently In Fig 9 are seen post stimulus histograms of the discharges of such a cell in response to stimulation of the median (A) and ulnar (C) nerves The stimulus artefact is indicated by the arrow The cell was also fired repetitively by the superficial radial nerve but not antidromically from the medial lemniscus The response to both the median and the ulnar nerve showed a tendency to discharge a train of spikes at fixed intervals but all discharges ceased after about 20 ms On local cuneate cooling to 26° C, however, the cell discharged with a slightly decreased initial latency to median volleys and somewhat longer latency to ulnar stimuli The most prominent change was however, that the discharges lasted much longer than at normal temperature, namely for more than 50 ms (Fig 9 B D) The same applied to the responses to superficial radial volleys Following rewarming of the cuneate region the discharge pattern returned to the control situation This is the behavior expected for a neurone involved in presynaptic depolarization matching both the prolonged and augmented dorsal column reflex and the presynaptic excitability changes observed (Fig 5 7)

A further indication that this type of neurone may be involved in presynaptic depolarization was its behavior to repetitive stimulation When recording from the same cell as shown in Fig 9 the cuneate region was cooled to 24° C The median nerve was stimulated at 1 and 20/s (Fig 10 A B) At the high rate of stimulation the latter part of the discharge disappeared corresponding to the abolition of the dorsal column reflex by repetitive stimulation at 10/s or higher Twelve of the twenty interneurones studied belonged to this category

Discussion

The reduction of synaptic transmission caused by local cooling of the cuneate region could be due to a failure of the transmission as such either by interference with conduction in preterminal fibres or by an impaired transmitter release mechanism or a reduced excitability of the postsynaptic membrane However an alternative explanation is that the inhibitory mechanisms present are enhanced and prolonged Since both presynaptic and post synaptic inhibition have been shown to be present in the dorsal column nuclei (Andersen *et al* 1964, Andersen Etholm and Gordon 1970) the susceptibility of both mechanisms to local cooling had to be investigated The inhibitory curves were indeed prolonged by local cooling When tested with *the double shock technique the inhibition lasted up to 200 ms and should only* affect transmission when the afferent volleys arrive with a frequency of 5/s or higher However if the conditioning volley initiates long lasting interneuronal discharge a certain small inhibitory effect may influence the transmission at lower rates of stimulation as well Because the synaptic transmission was so readily reduced even down to a frequency of 1/s it is likely that factors other than prolonged inhibition operate during cooling

Regarding the type of inhibition being susceptible the post synaptic inhibitory mechanisms studied were not affected to any significant degree by local cooling Conversely presynaptic depolarization was prolonged by local cooling and interneurones were also found that behaved in accord with the idea that presynaptic inhibition was enhanced by local cooling It is conceivable that this is the only type of inhibition that is prolonged during cooling

The effect of cooling is in accord with data from presynaptic depolarization in other areas notably the lower part of the spinal cord (Barron and Matthews 1938 Toennies 1938 1939) and the associated dorsal root reflexes (Brooks Koizumi and Malcolm 1955 Brooks and Koizumi 1956 Eccles Kozak and Magni 1961)

Whether the prolonged duration of the presynaptic inhibitory mechanisms has any physiological value is difficult to decide There is good evidence that presynaptic inhibition is involved in the efferent inhibitory control of sensory impulses from the skin (Gordon and Jukes 1964 a Andersen Etholm and Gordon 1970) One might assume that the survival value of retaining afferent inhibitory mechanisms at moderately reduced cord temperatures could have been a decisive factor However since consciousness is lost in man when the core temperature is reduced to 34° C (Fay and Smith 1941) the resistance of presynaptic depolarization and the corresponding inhibitory mechanisms to body temperature reduction seems to be of moderate importance

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Cholinesterase Activity in Rough and Smooth Brain Microsomal Membranes

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Abstract

HÄRKONEN, M., H. HELVE and H. TERÄVAINEN. *Cholinesterase activity in rough and smooth brain microsomal membranes.* Acta physiol. scand. 1972. *84.* 462—471.

Rat brain cortex was homogenized in 0.25 M or 0.32 M sucrose and sedimented at 10,000×g for 20 min. The supernatant was further separated into two subfractions by density gradient centrifugation in Cs⁺ containing sucrose. Electron microscopy, RNA/protein and phospholipid/protein indices showed that one of the subfractions was similar to the rough-surfaced (R) and the other to the smooth-surfaced (S) microsomal fractions separated from liver tissue by Dallner (1963). Cholinesterase (ChE) activity was studied by titration. Only 8—13 % of the total ChE activity was recovered in the 10,000×g supernatant and about 3—6 % in the subfractions. Both the R and S microsomal subfractions displayed ChE activity; however, the S subfraction had about three times as much activity per mg protein as the R subfraction. Activity due to nonspecific cholinesterase was 13 % of the total ChE activity in the R fraction and 7 % in the S fraction when the homogenate was prepared by homogenization with a clearance of 250 μm at 840 rpm. More vigorous homogenization increased the activities. Histochemical staining for AChE and ns ChE activities revealed reaction product adjacent to both R and S vesicles.

Electron microscopic histochemical studies have revealed that cholinesterase (ChE) activity inside the nerve cell is located either exclusively in the rough-surfaced membranes of the endoplasmic reticulum (e.g. Koelle and Foroglou-Kerameos 1965, Brzin, Tennyson and Duffy 1966, Lewis and Shute 1966) or in both the endoplasmic reticulum and the smooth-surfaced membranes of the Golgi complex (Eränkö, Rechardt and Hanninen 1967, Matsuura and Fujita 1968, Rechardt 1969). These differences can probably be explained on the basis of differences in the techniques, the animal species and the nervous tissue used. Moreover, histochemical techniques may have possible drawbacks, especially at the submicroscopic level, e.g. selective inhibition of enzymes by fixation and poor penetration of substrates or coupling agents to the enzyme site, which may interfere with the result.

When the intracellular distribution of ChE activity in nerve tissue was studied by centrifugation of brain homogenates, the largest part of the activity was found in the

fraction containing microsomal membranes (Toschi 1959, Holmstedt and Toschi 1959) The activity observed in mitochondrial fraction at first thought to be due to microsomal contamination (Aldridge and Johnson 1959), has been shown by sucrose density gradient fractionation to be due to nerve endings (synaptosomes) having about the same density as mitochondria (De Robertis *et al* 1962, Whittaker, Michaelson and Kirkland 1964)

Although there are several studies concerning the enzymic properties of brain microsomes the present work was undertaken to separate rough (R) and smooth (S) microsomal membranes using a procedure modified from Dallner (1963), characterize them in terms of RNA/protein and phospholipid/protein indices and describe their ChE activity The degree of purity of microsomal fractions was studied and an attempt made to locate the ChE activity within the different membranes of the R and S fractions with the electron microscope

Material and Methods

Preparation of microsomal fractions [illegible] decapitation were used in each experiment [illegible] n ice-cold 0 25 M or 0 32 M CO_2 free sucrose [illegible] or 10 % homogenate) and homogenized using [illegible] with two pestles giving clearances of either 150 or 250 μm at either 1 700 or 840 rev/min of the pestle, respectively Debris of the cell membr[illegible] sedi[illegible] 20 [illegible]

[illegible] was removed with a Pasteur pipette It was then placed in a 5 ml centrifuge tube diluted with 3 ml of water and brought up to the tube volume with 0 25 M sucrose Afte[illegible] 00 × *g* for 60 min at 2° C in Spinco Ti 50 rotor [illegible] e preceding one were called smooth (S) and to[illegible] homogenate was used the [illegible] C for

[illegible] *et al* [illegible]onucleic acid (RNA) was extracted and measured according to the principle of Ceriotti (1955) The orcinol colour reaction was developed in volumes of one tenth of the original method Ribose was employed as the standard and a factor of 3 76 was used to calculate the amount of RNA [illegible] roform methanol mixture and was [illegible] determi[illegible] (1957) Phosphorus was [illegible] phorous [illegible] rtlett (1959) The phos[illegible] 1963) [illegible] of phospholipid (Dallner

Measurement of cholinesterase activity The pellets were suspended by homogenization in 1 ml of 0 25 M sucrose solution made in CO_2-free distilled water and assayed at pH 8 0 and 25° C by automatic titration (Holmstedt Lundgren and Sjoqvist 1963) in a final volume of 5 1 to 5 5 ml

Acetylcholinesterase (E C 3 1 1 7) activity (AChE) was measured either with acetylcholine (ACh) as substrated and 10^{-5} M tetra[illegible] Co Ltd England) as inhib[illegible] 1956) or with acetyl methyl[illegible] specific cholinesterase (E.C[illegible] choline (BuCh) (Ord and [illegible] dimethylammonium phenyl) [illegible] land) (Bayliss and Todrick 1[illegible]

TABLE I Effect of two fractionation procedures on RNA/protein and P lipid/protein indices in brain microsomal subfractions

Procedure	Supernatant		R Pellet		S Pellet	
	RNA/Protein	P lipid/Protein	RNA/Protein	P lipid/Protein	RNA/Protein	P lipid/Protein
Brain						
— 0 25 M sucrose, 150 μm, 1700 rev/min	0 0415	0 165	0 158	0 304	0 053	0 515
— 0 32 M sucrose, 250 μm, 840 rev/min	0 0562	0 138	0 136	0 212	0 051	0 406
*Liver**						
— 0 25 sucrose Teflon, —			0 33	0 16	0 05	0 37

Rat cerebral cortices were homogenized in 0 25 or 0 32 *M* sucrose at 1700 or 840 rev/min in a homogenizer having a clearance of 150 or 250 μm between the pestle and the cylinder The values are the average of two separate fractionations
* Dallner 1963

(Mendel and Rudney 1943) In most cases the enzyme (100—500 μl) was preincubated with or without the specific inhibitor (5 μl) in 5 ml of 0 1 M KCl (CO_2 free) at pH 8 0 for 30 min in a separate closed test tube Thereafter the whole tube content was transferred to the reaction vessel and the substrate added in a final concentration of 6 mM The same solution after addition of eserine HCl in a final concentration of 10^{-4} M served as a blank (Easson and Stedman 1937) In some cases the consumption of 0 005 M NaOH was first recorded with enzyme and substrate and iso-OMPA and 284C51 were then added at 10 min time intervals and *enzyme activities calculated from the differences in the slopes Conditions were such as to* [illegible] and amount of enzymes [illegible] nal pellets were separately fixed for 2 5 h [illegible] l, purified Taab Laboratories England) [illegible] Sabatini Bensch and Barrnett 1963) The [illegible] washed in 0 25 M sucrose for 15 min to 12 h before final fixation for 1 5 h in 1 % OsO_4 buffered at pH 7 4 with phosphate Dehydration with a graded ethyl alcohol series was followed by embedding in Epon 812 (Luft 1961) *The sections used for routine electron microscopy were afterstained on grids with* lead citrate (Reynolds 1963) and the degree of purity of the pellets examined with the photomicrographs taken with a Philips EM 200 electron microscope

Histochemical technique for the demonstration of cholinesterases The ferro-ferricyanide method (Karnovsky and Roots 1964 Karnovsky 1964) was employed to locate the ChE activity within the membranes of the pellets AChE activity was demonstrated with acetylthiocholine iodide (Fluka AG Buchs) as substrate [illegible] tion for the inhibition of other ChE activity [illegible] with 10^{-5} M 284C51 was used to demonstrate [illegible] 10 to 60 min with either phosphate buffered 3 [illegible] 4° C and pH 7 4 except when unfixed specime[illegible] were rinsed for 10 min in 0 25 M sucrose transferred to the 1 % OsO_4 solution and processed as for routine electron microscopy (see above) Both the unfixed and fixed pellets were incubated for variable time ranging from 20 min to 4 h at 4° C at room temperature or at 37° C Preincubation for 30 min with the inhibitor was followed by incubation with the substrate Both inhibitors were used to test the validity of the reaction These test specimens were negative

Results

The purity of fractions The effect of vigorous and gentle homogenization is compared in Table I When rat liver was used as a control organ the separation and pellets were found to be similar to those obtained by Dallner (1963) There were no

major differences between the different fractionation methods used in the present work as judged either from electron micrographs or from RNA and phospholipid indices (Table I) Cross contamination estimated from electron micrographs of the pellets varied between 1 and 15 % In fractions containing mainly S membranes multivesicular bodies were also found (Fig 2) An occasional multivesicular body and a few small mitochondria could be seen in the fractions containing mainly R membranes (Fig 1) Some glycogen granules were also seen in both the R and S pellets The RNA/protein index of the R vesicles (0 147) was about three times that of the S vesicles (0 052) (Table I) The phospholipid/protein ratio was about twice as high in the S fraction (0 465) as in the R fraction (0 256) (Table I) These values are similar to those observed in liver fractionations except that the RNA/protein index in the liver R pellet was six times higher than in liver S pellet These results support the idea that the separation of rough and smooth surfaced microsomal membranes from brain was achieved and this separation appears similar to that obtained earlier with liver by Dallner (1963)

The ChE activity ACh was the substrate most rapidly hydrolyzed by rat brain cortex homogenate (Table II and III) Propiochylcholine (ProCh) was also fairly rapidly hydrolyzed and the hydrolysis was mainly 284C51 sensitive *i e* was due to AChE activity (Table II) BuCh on the other hand was hydrolyzed very slowly (Table II) and so were MeCh ($3\ 8\times10^{-3}$ μmol/mg protein/min) and BeCh ($2\ 5\times10^{-3}$ μmol/mg protein/min) ChE activity in the homogenate prepared with a loosely fitting (250 μm) pestle in 0 32 M sucrose was increased approximately 20 % if the sample was rehomogenized with a glass homogenizer This most probably was due to breakage of smaller structures than neurons *e g* axons intact synaptosomes smaller (glial) cells and nuclei which rendered more enzyme accessible to the substrate

Only 8—13 % of the total ChE activity of the homogenate was found in the $10\ 000\times g$ supernatant (Table II and III) Part of the microsomal membranes were apparently lost in the $10\ 000\times g$ pellet since electron micrographs showed both R and S vesicles in this fraction ChE activity in the $10\ 000\times g$ supernatant was approximately equally distributed between microsomal subfractions and the high speed supernatant (Table II and III) The activity remaining in the supernatant 43—87 %) presumably reflects the solubilization of the enzymes

The S membranes were about two times more abundant in brain microsomes than the R membranes if the calculation is based on protein content The more vigorous homogenization increased this ratio to 5 Both pellets regardless of the fractionation procedure contained all types of ChE activity studied (Table II and III The specific activities of the total ChE and AChE were however higher in S membranes than in R membranes The differences depended on the type of homogenization used for the preparation of the membranes the ratio varying between 2 4 and 3 The specific activities of the total ChE and AChE in the S subfraction were also somewhat higher than those of the homogenate However when ns ChE activity was measured or BuCh served as substrate the specific activities of membranes and homogenate were about the same In contrast to the homogenate the R membranes

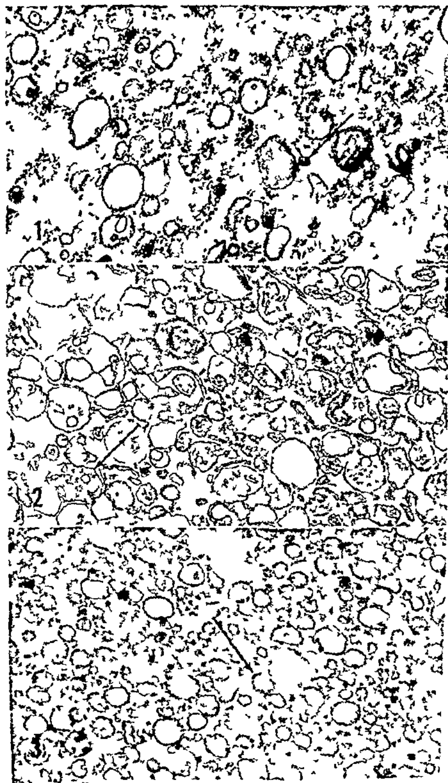

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TABLE II Cholinesterase activities in fractions of brain homogenate

Fraction	ACh			ProCh			BuCh
	Total ChE	AChE	ns ChE	Total ProChE	AChE	ns ChE	Total BuChE
	$\times 10^{-3}$ µmol/mg/min						
Homogenate							
— wet weight	7 20	6 20	0 77	4 60	3 70	0 78	0 61
— protein	62 8	54 5	6 7	41 4	34 0	7 2	5 5
— %	100	100	100	100	100	100	100
10 000 x g Supernatant							
— protein	20 0	18 8	1 6	14 2	12 3	1 9	1 9
— %	8 4	9 1	6 3	7 9	8 4	6.2	8 0
R Pellet							
— protein	22 8	20 6	4 6	36 3	33 5	2 8	4 2
— %	0 5	0 5	0 9	0 6	0 7	0 3	0 6
S Pellet							
— protein	67 0	60 6	5 0	55 2	51 7	4 1	4 1
— %	3 1	3 3	2 7	2 0	2 2	0 9	1 1
Soluble ChE in 10,000 x g Supernatant							
— %	57	58	43	67	65	80	79

Homogenization was performed in 0 25 M sucrose (25 % homogenate) with a homogenizer of 150 µm clearance at 1700 rev/min For enzyme analysis rehomogenization of the homogenate was performed [illegible]

hydrolyzed ProCh at a rate slightly higher than ACh This activity was mainly due to AChE activity

Histochemical demonstration of AChE and ns ChE showed some granular reaction product mainly adjacent to the tightly packed membranes of both R and S

Fig 1 Electron micrograph of a pellet separated by gradient ultracentrifugation in 0 32 M sucrose after homogenization for 2 min at 840 rev/min with a clearance of 250 µm The pellet consists mainly of free ribosomes and ribosome containing membranes but occasionally smooth vesicles are found (arrow) ×33,000

Fig 2 The pellet obtained after homogenization at 840 rev/min with a clearance of 250 µm followed by gradient ultracentrifugation in sucrose Membranes presumably originating from the smooth surfaced endoplasmic reticulum and the Golgi complex in addition to fragments of cell membranes of both the glial cells and the neurons are shown No free ribosomes are found within the pellet and ribosome containing membranes are also barely discernible Some of the membraneous structures bear some resemblance to multivesicular bodies (arrow) of intact neurons × 56 000

Fig 3 [illegible] d 10 M [illegible] embranes [illegible] the pre- [illegible] pellet was obtained in gradient ultracentrifugation after homogenization at 1700 rev/min with a clearance of 150 µm × 23.000

TABLE III Cholinesterase activities in fractions of brain homogenate

Fraction	Total ChE		AChE		ns ChE	
	Mean	SE	Mean	SE	Mean	SE
			$\times 10^{-4}$ µmol/mg/min			
Homogenate						
— wet weight	6.28	0.07	5.20	0.39	0.65	0.14
— protein	56.0	2.8	46.4	3.2	5.8	1.6
— %	100		100		100	
10 000 x *g* Supernatant						
— protein	18.0	1.8	16.4	1.3	1.87	0.36
— %	7.8	0.4	8.7	0.4	7.9	0.5
R Pellet						
— protein	31.8	2.6	28.1	1.9	4.0	0.77
— %	0.7	0.06	0.8	0.11	0.9	0.05
S Pellet						
— protein	81.6	12.1	74.9	11.8	5.6	1.4
— %	2.6	0.4	2.9	0.4	1.9	0.5
Soluble ChE in 10 000 x g Supernatant						
— %	57		58		64	

Homogenate was prepared in 0.32 M sucrose (25 % homogenate) with the aid of a homogenizer

vesicles (Fig 3) indicating that the activities analyzed by the biochemical procedures were indeed due to microsomal activity Although the histochemical method demonstrated the presence of ChE activity in the vesicle fractions the localization was clearly not as good as observed when the same method is applied to tissue blocks

Discussion

The fractionation of rat microsomes in Cs containing sucrose produces two distinct membrane fractions which can be shown by chemical and electron microscopic characterization to be similar to the R and S subfractions described in liver by Dallner and Ernster (1968) The R vesicles presumably originate exclusively from the rough surfaced endoplasmic reticulum of the cells of the brain cortex The S vesicles are possibly derived from several different sources, they probably originate mainly from the smooth surfaced endoplasmic reticulum and the Golgi complex and to a smaller degree from remnants of the cell and axonal membranes The special loosely fitting homogenized and 0.32 M sucrose were used to prepare microsomes in the hope of getting most of the nerve endings and small glial cells in the 10 000×g pellet. Electron microscopy of this pellet showed indeed several intact nerve endings, red blood cells, leukocytes endothelial cells and glial cells However since glial

cells are more numerous in the cerebral cortex than neurons many of them presumably have been broken down during homogenization and part of their constituents may also be included in both microsomal subfraction Some other cells, *e g* white blod cells and endothelial cells are presumably of little importance as possible source of contamination

At best only about 15 % of the total ChE and 13 % of AChE activities were found in the 10 000×*g* supernatant (10 % homogenate) This is less than that reported by Toschi (1959) and Holmstedt and Toschi (1959), who found most of the AChE activity in the microsomal fraction De Robertis *et al* (1962) found 39 % of the AChE activity in microsomes These differences in the ChE activity of the supernatant can probably be attributed mainly to the degree of breakage of nerve endings, axons, dendrites and glial cells These breakages may be less frequent with our technique than with those previously used In our system the amount of ChE lost to the 10 000×*g* pellet was probably greater than those reported by De Robertis *et al* (1962) and Whittaker *et al* (1964) who sedimented the nuclear pellet first, and the nerve endings and mitochondria afterwards In our modification part of the micro somal vesicles were brought down in the 25 % homogenate into the 10 000×*g* pellet because of a higher centrifugal force in a more concentrated homogenate than might have been obtained in those cases in which centrifugation was done in two steps This seems also to be supported by the fact that the 25 % homogenate in 0 32 M sucrose had even less supernatant AChE activity than when the 10 % homogenate was used (8 % vs 13 %) Because the main purpose of our work was to separate R and S vesicles this loss was accepted since the method used presumably simultaneously diminished the contamination of fractions by other ChE containing membranes

The low ns ChE activity found in the R and S pellet (13 and 7 % of the total ChE activity after gentle homogenization respectively) could be due to the glial contamination This enzyme is predominantly present in glial cells but has also been found in some neurones and non nervous structures (Koelle 1963 Harkonen 1964, Torack and Barrnett 1962)

The rather low specific AChE activity of the R pellet compared to the S pellet does not agree with earlier histochemical observations, where the intraneuronal AChE activity was localized mainly in the rough endoplasmic reticulum (see Introduction) There are several possible explanations for this apparent discrepancy The fixation prior to histochemical tests with intact cells may have inactivated the ChEs in other parts more than in the rough endoplasmic reticulum On the other hand, the fixation may have caused some enzyme which forms part of the relatively soluble ChEs, to be bound to the membranes The relatively high specific AChE activity of the S membranes could be due to contamination by other AChE containing mem branes like remnants of plasma and axonal membranes which would not be detected in electron micrographs The contamination of R membranes by other membranes appeared minimal on electron micrographs, further the histochemical reaction product was located on the R vesicles, therefore, we are inclined to believe that the low specific activity of the R pellet was mainly due to sol[illegible] the

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Influence of Plasma Hypertonicity on Blood Viscosity Studied in Vitro and in an Isolated Vascular Bed

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Abstract

RASMUSSEN, S NYBO *Influence of plasma hypertonicity on blood viscosity studied in vitro and in an isolated vascular bed* Acta physiol scand 1972 *84* 472—481

The effects of plasma hypertonicity on blood viscosity have been examined *in vitro* as well as in a *vascular bed* NaCl was added to ox blood to produce plasma osmolalities up to about 1000 mosm/kg The isolated rabbit ear was perfused at 70 and 30 mm Hg Apparent viscosities of blood relative to plasma were calculated from the corresponding flow ratios (*in vivo* viscosity Relative viscosities *in vitro* of the perfusates were determined by means of a cone plate viscometer Blood viscosity measured *in vivo* as well as *in vitro* was significantly increased by increasing osmolality Maximal increments (almost 100 per cent) were observed between 700 and 900 mosm/kg Plasma viscosity and vascular resistance was not influenced by hypertonicity Viscosities *in vivo* were generally lower than *in vitro*—the discrepancy was more pronounced at 70 than at 30 mm Hg and was greater in isotonic than in hypertonic blood The effect of hypertonicity on blood viscosity is explained mainly by a reduced deformability of the hypertonically crenated red cells It seems likely to explain the inverse relationship between renal medullary blood flow and medullary hypertonicity (Thurau *et al* 1960) as caused partly by passive mechanisms involving blood viscosity changes with varying osmolality

The influence of increased plasma osmolality on the rheologic properties of blood has been studied *in vitro* by different workers The viscosity of whole blood and of suspensions of erythrocytes in electrolyte solutions is considerably and reversibly increased by the addition of NaCl mannitol or dextrose whereas the addition of urea has no such effect (Rand and Lacombe 1964 Meiselman *et al* 1967, and Ham *et al* 1968) Schmid Schonbein and Wells (1969) and Schmid Schonbein *et al* (1969) demonstrated that the viscosity of blood with crenated red cells at low rates of shear is reduced because of abolished aggregation whereas at high rates of shear the viscosity is increased due to reduced viscous deformability (fluidity) of the shrunken red cells

Concerning normal *isotonic* blood different investigators have compared *in vitro* measured viscosities with the apparent relative viscosities determined as flow ratios in isolated vascular beds Skovborg *et al* 1968 using the isolated rabbit ear as a biological viscometer found that the apparent relative viscosity is lower than the in vitro measured relative viscosity of blood A similar discrepancy

between blood viscosity *in vivo* and *in vitro* was observed by Djojosugito *et al* (1970) using the vascular bed of the calf muscles in the cat With decreasing flow rate apparent viscosity increases, however, barely reaching the figures obtained *in vitro* at even the highest shear rates These results stress that figures for blood viscosity obtained in viscometers may be misleading for evaluation of the biological situation

The purpose of the present study was to examine the effects of plasma hypertonicity on blood viscosity *in vitro* as well as in an isolated *vascular bed* With special reference to the renal medullary vessels hypertonicity was produced by adding NaCl in amounts appropriate for obtaining total plasma osmolalities up to about three to four times the normal level The maximally dilated vascular bed of the isolated rabbit ear was used as a biological viscometer in a perfusion setup Preliminary results have been published (Nybo Rasmussen 1970)

Methods and Materials

Preparation of the rabbit ear Rabbits weighing 2—4 kg were anesthetized with urethan 1 5 g/kg i v In order to reduce vascular tone phenoxybenzamine 200 μg/kg was injected i p The preparation of the ears was started about 30 min later Following i v injection of 2500 I U of heparin the central artery of both ears was dissected free and cannulated with a polyethylene catheter (i d = 0 58 mm) Immediately after cannulation the ears were flushed through with 37° C warm 0 9 % NaCl containing 70 I U of heparin and 10 μg of phenoxybenzamine per ml The rabbit was killed by i v administration of an overdosage of pentobarbital and the ears were cut off near the base with a scalpel Both ears were then flushed again with saline in order to remove all rabbit plasma and blood from the vascular system Immediately after weighing one ear was transferred to the perfusion set up while the other was kept in a refrigerator at 5° C for later use normally the next day This storage did not alter the flow characteristics of the ear

The perfusion set up is a modification of that described by Skovborg *et al* (1968) (Fig 1) The ear is placed on a slightly tilted plexiglass table from which the venous effluent is collected in a light plastic cup (net weight 0 8 g) for continuous weight recording by means of a Statham pressure transducer (type PM5TC) providing a highly sensitive balance exhibiting perfect linearity The perfusion pressure is measured by means of a Statham transducer (type P23AA Both transducer signals are recorded on a two channel potentiometer recorder (Servogor Goerz electro Vienna Austria) The plastic cup is emptied by suction everytime an electromagnetic clamp is opened The opening and closing of the clamp is operated automatically from the recorder by means of two microswitches being activated directly by the weight recording pen

All measurements were performed at 37° C The whole set up was placed in a lucite box in which the temperature was kept at 37±0 5 C and the reservoirs were provided with water caps through which water at 37±0 2° C was circulated

Perfusion media Freshly drawn venous ox blood was stabilized with EDTA 2Na 1 mg/ml After some preliminary experiments perfusion with whole blood was given up because of clogging of the vascular system In the experiments here reported the buffy coat was removed by careful suction following centrifugation and removal of plasma The red cells were then resuspended in their plasma and the whole procedure was repeated twice more Finally the hematocrit was adjusted to 40 and the blood was added phenoxybenzamine 1 mg/100 ml which had been found to keep the vascular bed maximally dilated From this batch two aliquots were taken To the one was slowly added 18 % NaCl in a volume sufficient to reach the desired total osmolality To the other was added the equal volume of 0 9 % NaCl in order to obtain identical red cell numbers Apart from the dilution of the hypertonic plasma caused by erythrocyte water plasma protein concentrations should become identical too Part of both the isotonic and the hypertonic portion was centrifugated to get the pure suspension media Available for each experiment were now four perfusion media isotonic plasma I isotonic blood II hypertonic plasma (III), and hypertonic blood (IV)

Analyses Hematocrit hemoglobin concentration red cell number plasma osmolality and viscosity in vitro were determined in samples of the perfusion media Hematocrits were determined in a microhematocrit centrifuge at 14 000 × g for 5 min Hemoglobin conc

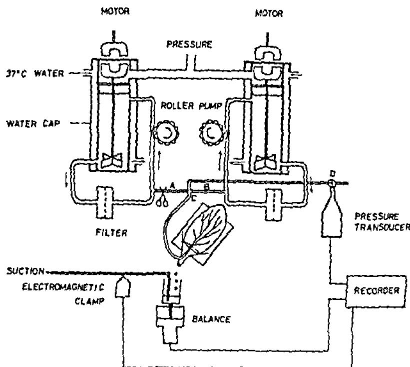


Fig 1 The experimental set up used for constant pressure perfusion of the isolated rabbit ear Two 50 ml plexiglass reservoirs one for plasma and one for blood are connected to an adjustable constant pressure delivery system Roller pump and stirrer secure recirculation Rapid shifts of perfusate are done by moving a clamp between A and B Perfusion pressure is measured at point C A short catheter (about 2 cm) connects C with the central artery The ear can be flushed from a syringe at the stopcock D The venous outflow is continuously determined by means of a balance system (for further details see methods)

were measured photometrically with the cyanmethemoglobin method The osmolalities were determined from the freezing point depression of plasma measured by a commercial apparatus (Knauer Berlin)

Viscosity *in vitro* of the perfusion media I—IV was determined in a Wells Brookfield micro cone-plate viscometer model LVT Brookfield Engineering Laboratories Inc Stoughton Mass) at 37° C using 1 ml samples The absolute viscosity in centipoise was calculated from the measured shear stress at the following shear rates 230 115 46 and 23 sec $^{-1}$

Statistical analysis was carried out according to the Student s t test using the 5 per cent level as a criterion of significance

Experimental procedure

For perfusion of each ear blood obtained from a single x was used for preparing the above mentioned perfusates I—IV Each ear preparation was perfused at constant pressure(s) with the 4 perfusates successively (Fig 2) In order to diminish the influence of inevitable alterations in the preparation due to varying omolality of the perf n media a perfusion with a certain erythrocyte suspension was always preceded by a perfusion with the corresponding suspension medium Blood viscosity was calculated as a *relative apparent viscosity* from the respective flow values for suspension (blood) and suspension medium (plasma) When enough plasma was available a second perfusion with plasma was carried out as a control following the perfusion with blood

The duration of each perfusion experiment was on an average 1 1/2 h The average ear weight before starting the perfusions was 21 g The perfusions were generally accompanied

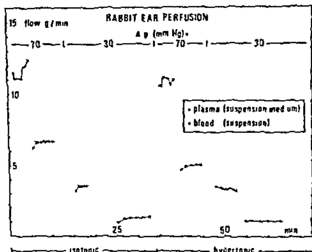


Fig 2 The time course of a single rabbit ear perfusion The perfusion pressures used are shown at the top Each point represents the mean flow rate for the period (or part of the period) between 2 emptyings of the outflow collecting cup The flow is calculated from the slope of the accumulated weight curve The dotted lines indicate the flow values used for calculation of the relative apparent viscosities ('in vivo')

by an increase in weight of the isolated ear, which at the end of the experiments appeared somewhat oedematous The isotonic perfusions were on an average accompanied by a 15 per cent increase in weight, the subsequent hypertonic perfusions by a further 25 per cent increase relative to the initial weight

The experiments were divided into four groups (1—4) according to the level of hyperosmolality The mean ($\pm$SE) osmolalities of the hypertonic media in the four groups were 414 ± 3, 536 ± 13, 736 ± 6, and 977 ± 12 mosm per kg The isotonic media showed nearly identical osmolalities in the four groups (mean$\pm$SE of all groups 300 ± 2 mosm per kg)

Results

The experiments were carried out on 35 different rabbit ear preparations 24 preparations were perfused at both 70 and 30 mm Hg (Fig 2) 11 preparations only at 70 mm Hg

The average *perfusion rates* (in g/min and 100 g initial ear weight) obtained with the 4 perfusates at 70 and 30 mm Hg are given in Table A for the 4 groups of experiments The mean of all groups (right column) was calculated after having performed an analysis of variance A subsequent comparison revealed no significant differences between the four groups regarding flow rates of the isotonic perfusates The flow rates of the increasingly hypertonic perfusates of group 2 3 and 4 were compared with the moderately hypertonic perfusates of group 1 The values for blood of the more hypertonic groups were significantly lower than of group 1, whereas the values for plasma showed no significant differences This last observation was taken as an evidence that in this preparation vascular resistance is not influenced by changes in osmolality itself This was further supported when isotonic and hypertonic plasma flows within each group were compared Except for one case (group 3 at $\Delta p = 70$ mm Hg) none of the differences between isotonic and hypertonic plasma flows within the same group were statistically significant at the 5 per cent level

The average *viscosities* in centipoise (cp) of the four perfusates measured *in*

TABLE A Perfusion rates in gram per minute and 100 g ear weight The numbers 1—4 refer to the four groups of experiments separated according to the different levels of hypertonicity The results are given as mean values ± SE The number of observations in each group are given in parenthesis

Group	1	2	3	4	mean of all groups
Δ P = 70 mm Hg	49 4±7 1 (8)	37 9±8 2 (7)	31 6±5 5 (10)	51 4±6 1 (9)	42 3±3 5 (34)
Plasma					
isotonic	57 2±5 2 (8)	43 3±6 6 (7)	49 2±5 7 (10)	59 0±4 9 (9)	52 4±2 9 (34)
hypertonic	30 5±3 4 (8)	24 6±3 9 (7)	21 5±2 6 (10)	29 5±3 3 (9)	26 4±1 7 (34)
Blood					
isotonic	27 4±2 1 (8)	18 7±2 5 (7)	16 3±1 5 (10)	17 9±1 1 (9)	
Δ p = 30 mm Hg					
Iso	18 4±1 9 (8)	20 7±4 6 (7)	10 2±2 0 (3)	16 7±1 7 (10)	16 8±1 2 (23)
Plasma					
Hyper	17 1±1 6 (8)	12 8±2 8 (6)	12 3±1 5 (3)	13 3±2 4 (9)	14 2±1 2 (26)
Iso	7 8±0 9 (8)	7 8±0 6 (2)	4 7±1 1 (3)	7 8±0 9 (10)	7 4±0 5 (23)
Blood					
Hyper	5 7±0 6 (8)	3 7±0 6 (6)	3 3±0 2 (3)	3 9±0 5 (9)	

vitro at shear rates 230 115, 46 and 23 s⁻¹ are presented in Fig 3 for the 4 groups of experiments All perfusates plasma as well as blood behaved as non Newtonian liquids showing increasing viscosity with decreasing shear rate With increasing shear rate the viscosities approached minimum values at shear rate 230 s⁻¹ Fig 3 gives the general impression that hypertonicity produced by NaCl does not influence the viscosity of plasma whereas blood viscosity is significantly increased A statistical comparison carried out within each group at all shear rates between values for isotonic and hypertonic plasma viscosity revealed no significant differences except for one case (group 2 at shear rate 230 s⁻¹ Concerning blood isotonic and hypertonic values in group 1 were not statistically different at the 5 per cent level except for shear rate 230 s⁻¹ In the groups 2 3 and 4 statistically significant differences between isotonic and hypertonic values were observed in all cases except for a few values obtained at low shear rates (these are marked with an asterisk in the figure)

The fact that hypertonicity at the actual levels produced by adding NaCl neither influenced plasma viscosity *in vitro* nor seemed to influence vascular resistance in the rabbit ear preparation permitted the use of the relative viscosity values (blood to plasma, see under methods) for comparison of the effects of different levels of hypertonicity on the viscosity of blood Average values from all experiments are presented in Fig 4 The isotonic values (unshaded columns) serve as a control for the hypertonic ones (cross hatched columns At bottom is shown the mean values of osmolarity erythrocyte number and hematocrit

In each group the *erythrocyte numbers* (as well as the concentrations of hemoglobin) of the isotonic and the hypertonic samples were closely identical This was

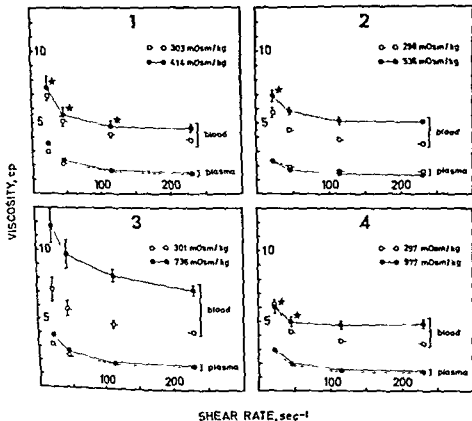


Fig 3 Absolute viscosities determined in vitro by means of a Wells Brookfield micro cone-plate viscometer The numbers 1—4

taken as evidence that the samples were comparable Furthermore, no significant hemolysis was observed following the preparation of the perfusates

The *hematocrits* of the isotonic samples were closely identical (mean ± SE of all groups 37 8 ± 0 2 per cent) The hematocrit of the hypertonic samples were significantly lower than the isotonic values in each group and were decreasing with increasing hypertonicity (range 33 4 (group 1) — 24 4 (group 4) The fall in hematocrit from the original of 40 per cent to around 38 per cent in the isotonic blood perfusates was caused by the addition of a volume of isotonic saline equal to the volume of hypertonic saline used in preparation of the hypertonic blood perfusates

It appears clearly from Fig 3 and 4 that the relative blood viscosity measured *in vitro* as well as '*in vivo*' is increased by increasing osmolality The phenomenon seems to reach a maximum at an osmolality between 700 and 900 mosm per kg In group 4 the fractional increments in viscosity from isotonic to hypertonic blood

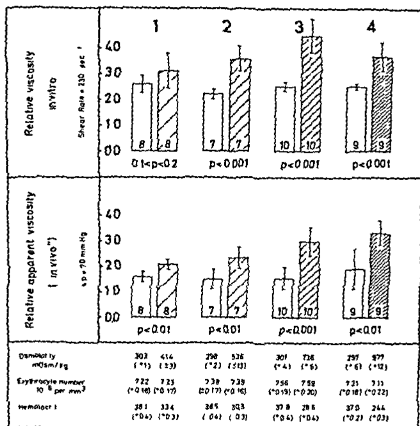


Fig 4 Relative viscosities (blood to plasma) for the four groups of experiments (1—4) Each column represents the mean value of n observations (n is given in the lower part of the column) The isotonic values (unshaded) serve as controls for the hypertonic ones (cross hatched) The vertical bars indicate the 95 per cent confidence limits of the calculated mean value Figures in the lower part are mean values ± SE

are relatively lower than those observed in group 3 independent of the method for the determination

Discussion

Evaluation of the rabbit ear preparation as a biological viscometer The suitability of the rabbit ear preparation as a biological viscometer has been demonstrated by Skovborg *et al* (1968) When a vascular bed is used as a viscometer, vascular dimensions must remain constant In this preparation constant and reproducible flow values could be obtained with a certain perfusate at a certain perfusion pressure Furthermore the addition of common vasodilating agents (acetylcholine, isoproterenol and nylidrin) did not increase flow

Comparing isotonic and hypertonic perfusates the possible effect on vascular resistance of hypertonicity itself must be considered too Mellander *et al* (1967), Gazitua *et al* (1969) and others have demonstrated a vasodilating effect of intravascularly administered hyperosmotic solutions A similar effect of hyperosmolality

itself was not observed in the present preparation (Table V), indicating that the vessels were already completely relaxed

Viscosities measured in vitro Viscometry *in vitro* on the different perfusates revealed different interesting details (Fig 3) Corresponding isotonic and hypertonic plasmas showed nearly identical viscosities although erythrocyte water lowered the protein concentration in the hypertonic plasmas compared to the isotonic plasmas

All perfusates exhibited non Newtonian viscosity (increasing as shear rate decreases) This finding is in agreement with the observations of other investigators (Wells and Merrill 1961 Rand *et al* 1964) However non Newtonian behaviour of plasma may be an artifact due to the air fluid interface in the cone plate viscometer (Merrill 1965, Meiselman 1971) In the case of isotonic perfusates the measured viscosities of blood and plasma were affected proportionally by variations in shear rate, which means that the calculated relative viscosity (blood to plasma) appeared to be shear rate independent The observed shear rate dependence of the viscosity of hypertonic blood however, seemed relatively less pronounced than that of all other perfusates This agrees with the observations of Murphy (1967) who described the viscosity of the blood in hypertonic serum as more Newtonian than in normal serum Schmid Schonbein and Wells (1969) published similar blood viscosity profiles (viscosity versus shear rate) showing an intersect between the curve of isotonic blood and that of hypertonic blood (522 mosm/kg produced by NaCl) at shear rate 1 sec^{-1}

Comparison between in vitro and in vivo obtained viscosities A comparison between relative viscosities of isotonic blood is shown in Fig 5 As mentioned above relative viscosity of isotonic blood *in vitro* proved to be shear rate independent Shear rate 230 sec^{-1} was chosen here because the accuracy of the viscosity determination is the greater the higher the shear rate It appears from Fig 5 that the values obtained *in vivo* are significantly higher at perfusion pressure 30 mm Hg than at perfusion pressure 70 mm Hg It is seen too that at perfusion pressure 30 mm Hg the *in vitro* results agree well with the values obtained *in vivo* while at perfusion pressure 70 mm Hg — a physiologically more relevant value — the *in vivo* results are significantly lower than the values obtained *in vitro* This is in accordance with the findings of Djojosugito *et al* (1970)

Axial migration of red cells in Poiseuille flow may explain why the relative apparent viscosity of blood is dependent on perfusion pressure Goldsmith (1968 pointed out that while rigid particles do not migrate axially deformable particles (drops) migrate axially at a rate which increases with increasing particle size velocity gradient (and hence deformation) and radial distance from the tube axis In smaller vessels axial migration results in significantly lowered apparent viscosity (Fåhraeus and Lindqvist 1931 Prothero and Burton 1962 and others) while in larger vessels—and presumably in the cone plate viscometer—the effect is less pronounced It is likely that a diminution of the perfusion pressure in the rabbit ear—although accompanied by some reduction of the vascular dimensions

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From the Department of Clinical Physiology and the Burn Center, University Hospital Uppsala Sweden

Proton- and Carbamino-linked Oxygen Affinity of Normal Human Blood

By

L GARBY M ROBERT[1] and B ZAAR

Received 30 September 1971

Abstract

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estimated and found to be dependant on the oxygen saturation level The values for the proton linked oxygen affinity and the carbamino-linked oxygen affinity were found by applications of Wyman s reciprocal relations and 2 3-diphosphoglycerate binding data to give good estimates of the oxygen linked proton binding and the oxygen linked carbamino binding The data show that *the oxygen linked carbamino reaction takes place mainly during the binding of the first 2 oxygen* molecules with a value of about 0 25 mol per mol of oxygen The proton release accompanying this reaction was estimated to be 1 3—1 7 mol per mol

One of the central functions of the blood is to transfer oxygen from the lungs to the tissues, carbon dioxide from the tissues to the lungs and protons from the tissues to the kidneys The transport system is unique in the sense that important changes in the affinity of the blood for these components take place during the actual exchange in the capillaries These changes are mediated through allosteric linkage of the transported molecules and ions to the haemoglobin molecule and the result is an augmentation of the exchange capacity of the blood for these same components

The molecular and biochemical reaction mechanisms of the allosteric effects in the hemoglobin molecule have been elucidated to a considerable extent during the last few years (see for example Perutz 1970) In contrast the physiological importance of the interactions between the four main ligands *i e* protons, carbon dioxide, oxygen and 2,3 diphosphoglycerate (2,3 DPG), of which the first 3 ligands are also

[1] In receipt of a grant from the Scholarship Foundation of the Swiss Academy of Medical and Biological Sciences.

transported components, has not yet been worked out. In fact, very elementary considerations of the properties of the transport system show that fundamental physiological data are still lacking. Specifically, the quantitative effect of protons and of carbon dioxide on the oxygen affinity of normal blood at different degrees of oxygen saturation has not yet been clarified. Results of such measurements form the core of the present communication.

Knowledge of the oxygen linked proton binding and of the oxygen linked carbamino binding as they occur when the monomers (subunits) of the hemoglobin molecule become successively oxygenated, is fundamental to the understanding of the molecular basis of the hemoglobin function. It will be shown in the present paper, by application of the general theory of linked functions (Wyman 1948 and 1964) to the data, that the experimentally determined proton and carbamino-linked oxygen affinity can, in fact, be interpreted numerically in terms of proton and carbamino binding during oxygenation of the hemoglobin molecule. For this reason, and in order to delineate precisely the relation between the present data and previous estimates of the so called Bohr coefficient and between the present data and estimates of the oxygen linked proton binding, it is necessary to develop in some detail the application of the theory of linked functions to the system.

Application of the theory of linked functions to the system of hemoglobin, oxygen, protons, carbon dioxide and 2,3-DPG in the erythrocytes

Among the possible general relations between the 5 components in the system we need to consider the following three only

$$\log pO_2 = f_1(\bar{O}_2, \log pCO_2, \log a_{H^+}, \log a_{DPG}) \quad (1)$$

$$\log a_{DPG} = f_2(\bar{O}_2, \log pCO_2, \log a_{H^+}, \log pO_2) \quad (2)$$

$$\bar{H}^+ = f_3(\bar{O}_2, \log pCO_2, \log a_{H^+}, \log a_{DPG}) \quad (3)$$

where a_{H^+} and a_{DPG} are the activities of protons and 2,3 DPG and $\bar{O}_2$ and $\bar{H}^+$ are the *numbers of moles* of oxygen and protons bound, respectively, and on the average to one hemoglobin monomer, *i.e.* $\bar{O}_2$ is the oxygen saturation fraction. The use of logarithms of the activities and the partial pressures in these equations makes it possible to introduce directly the reciprocal relations of Wyman (*vide infra*).

We develop first eq. (1). When the experimental observations of the oxygen tension are performed at constant oxygen saturation, *i.e.* constant $\bar{O}_2$, eq. (1) can be written after total differentiation and division by $d\log a_H$ as[1]

$$\left(\frac{d\log pO_2}{d\log a_{H^+}}\right)_{\bar{O}_2} = \left[\frac{\partial \log pO_2}{\partial \log a_{H^+}}\right]_{pCO_2, a_{DPG}} + \left[\frac{\partial \log pO_2}{\partial \log pCO_2}\right]_{a_H, a_{DPG}} \times \left(\frac{d\log pCO_2}{d\log a_H}\right) + \left[\frac{\partial \log pO_2}{\partial \log a_{DPG}}\right]_{a_{H^+}, pCO_2} \times \left(\frac{d\log a_{DPG}}{d\log a_{H^+}}\right) \quad (4)$$

[1] Variables on the right hand side of this and subsequent equations are defined not only by their own subscripts but also by the subscripts of the variable on the left hand side of the equation.

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We develop first eq (*1*) When the experimental observations of the oxygen tension are performed at constant oxygen saturation, *i e* constant $\bar{O}_2$, eq (1 can be written after total differentiation and division by dlog a_{H^+}, as[1]

$$\left(\frac{d\log pO_2}{d\log a_{H^+}}\right)_{O_2} = \left[\frac{\partial \log pO_2}{\partial \log a_{H^+}}\right]_{pCO_2\ a_{DPG}} + \left[\frac{\partial \log pO_2}{\partial \log pCO_2}\right]_{a_{H^+}\ a_{DPG}} \times \left(\frac{d\log pCO_2}{d\log a_{H^+}}\right) + \left[\frac{\partial \log pO_2}{\partial \log a_{DPG}}\right]_{a_{H^+}\ pCO_2} \times \left(\frac{d\log a_{DPG}}{d\log a_{H^+}}\right) \quad (4)$$

[1] Variables on the right hand side of this and subsequent equations are defined not only by their own subscripts but also by the subscripts of the variable on the left hand side of the equation

The left side of eq. (4) is identical to the classical Bohr coefficient if the change in $\log a_{H^+}$ is accomplished solely by a change in pCO_2. The effect on the oxygen affinity obtained in this way thus originates from three sources, i.e. the three terms on the right hand side of eq. (4). Since the uptake and release of protons and carbon dioxide in the capillaries are not in general coupled, the significance of the classical Bohr effect is not obvious. Experimental observations of the oxygen tension at fixed $\bar{O}_2$ can however be performed at constant pCO_2 so as to give

$$\left(\frac{d\log pO_2}{d\log a_{H^+}}\right)_{\bar{O}_2\ pCO_2} = \left[\frac{\partial\log pO_2}{\partial\log a_{H^+}}\right]_{a_{DPG}} + \left[\frac{\partial\log pO_2}{\partial\log a_{DPG}}\right]_{a_{H^+}} \times \left(\frac{d\log a_{DPG}}{d\log a_{H^+}}\right) \tag{5}$$

Furthermore, eq. (1) can be developed in analogy with eq.s (4) and (5) to give at constant $\bar{O}_2$ and constant $\log a_{H^+}$,

$$\left(\frac{d\log pO_2}{d\log pCO_2}\right)_{\bar{O}_2\ a_{H^+}} = \left[\frac{\partial\log pO_2}{\partial\log pCO_2}\right]_{a_{DPG}} + \left[\frac{\partial\log pO_2}{\partial\log a_{DPG}}\right]_{pCO_2} \times \left(\frac{d\log a_{DPG}}{d\log pCO_2}\right) \tag{6}$$

Two new coefficients thus appear, i.e. one for the protonlinked oxygen affinity and one for the carbamino linked oxygen affinity. Substitution in eq. (4) of the expressions

$[\partial\log pO_2/\partial\log a_{H^+}]_{\bar{O}_2\ pCO_2\ a_{DPG}}$ and $[\partial\log pO_2/\partial\log pCO_2]_{\bar{O}_2\ a_{H^+}\ a_{DPG}}$

from eq.s (5) and (6), respectively, gives

$$\left(\frac{d\log pO_2}{d\log a_{H^+}}\right)_{\bar{O}_2} = \left(\frac{d\log pO_2}{d\log a_{H^+}}\right)_{pCO_2} + \left(\frac{d\log pO_2}{d\log pCO_2}\right)_{a_{H^+}} \times \left(\frac{d\log pCO_2}{d\log a_{H^+}}\right)$$
$$- \left[\frac{\partial\log pO_2}{\partial\log a_{DPG}}\right]_{pCO_2\ a_{H^+}} \times \left(\frac{d\log a_{DPG}}{d\log a_{H^+}}\right) \tag{7}$$

so that the numerical value of the contribution of changes in $\log a_{DPG}$ can be estimated.

We next consider eq. 2. At constant values of $\bar{O}_2$ and $\log pO_2$ we have, in analogy with (5) and (6), that

$$\left(\frac{d\log a_{DPG}}{d\log a_{H^+}}\right)_{O_2\ pO_2} = \left[\frac{\partial\log a_{DPG}}{\partial\log a_{H^+}}\right]_{pCO_2} + \left[\frac{\partial\log a_{DPG}}{\partial\log pCO_2}\right]_{a_{H^+}} \times \left(\frac{d\log pCO_2}{d\log a_{H^+}}\right) \tag{8}$$

We finally consider eq. (3). At constant $\log a_{H^+}$ we have, after total differentiation and division by $d\bar{O}_2$

$$\left(\frac{d\bar{H}^+}{d\bar{O}_2}\right)_{a_{H^+}} = \left[\frac{\partial\bar{H}}{\partial\bar{O}_2}\right]_{pCO_2\ a_{DPG}} + \left[\frac{\partial\bar{H}^+}{\partial\log pCO_2}\right]_{O_2\ a_{DPG}} \times \left(\frac{d\log pCO_2}{d\bar{O}_2}\right)$$
$$+ \left[\frac{\partial\bar{H}^+}{\partial\log a_{DPG}}\right]_{O_2\ pCO_2} \times \left(\frac{d\log a_{DPG}}{d\bar{O}_2}\right) \tag{9}$$

The left hand side of eq. (9) is the oxygen linked proton binding at fixed $\log a_{H^+}$ sometimes called the Haldane coefficient. Thus at constant $\log a_{H^+}$, the oxygen-linked protons come from three sources: those at constant pCO_2 and constant a_{DPG} but also those from the oxygen linked carbamino binding and from the oxygen-

linked 2,3 DPG binding If, in addition to constant log a_{H^+}, logpCO_2 is also kept constant, we obtain

$$\left(\frac{d\overline{H}^+}{d\overline{O}_2}\right)_{a_{H^+}, pCO_2} = \left[\frac{\partial\overline{H}^+}{\partial\overline{O}_2}\right]_{a_{DPG}} + \left[\frac{\partial\overline{H}^+}{\partial \log a_{DPG}}\right]_{\overline{O}_2} \times \left(\frac{d\log a_{DPG}}{d\overline{O}_2}\right) \tag{10}$$

In the following, we shall make use of the reciprocal relations (Wyman 1948 and 1964) given below

$$\left[\frac{\partial \log pO_2}{\partial \log a_{H^+}}\right]_{\overline{O}_2, pCO_2, a_{DPG}} = -\left[\frac{\partial\overline{H}^+}{\partial\overline{O}_2}\right]_{a_{H^+}, pCO_2, a_{DPG}} \tag{11}$$

$$\left[\frac{\partial \log pO_2}{\partial \log a_{DPG}}\right]_{\overline{O}_2, pCO_2, a_{H^+}} = -\left[\frac{\partial\overline{DPG}}{\partial\overline{O}_2}\right]_{a_{H^+}, pCO_2, a_{DPG}} \tag{12}$$

$$\left[\frac{\partial \log pO_2}{\partial \log pCO_2}\right]_{\overline{O}_2, a_{H^+}, a_{DPG}} = -\left[\frac{\partial\overline{CO}_2}{\partial\overline{O}_2}\right]_{a_{H^+}, pCO_2, a_{DPG}} \tag{13}$$

$$\left[\frac{\partial\overline{H}^+}{\partial \log a_{DPG}}\right]_{\overline{O}_2, a_{H^+}, pCO_2} = \left[\frac{\partial\overline{DPG}}{\partial \log a_{H^+}}\right]_{\overline{O}_2, pCO_2, a_{DPG}} \tag{14}$$

Methods

Blood from 23 healt[illegible]
collected [illegible]
at two dif[illegible]
and with [illegible]
same sam[illegible]
respective [illegible]

[illegible] dissociation curves were run in a Radiometer DCA 1 apparatus, the principles of which have been described in detail by Duvelleroy *et al* (1970) This method has been shown to give results which are identical with those obtained by the mixing technique (Bellingham and Lenfant 1971) In the Radiometer DCA 1 [illegible]
tion proced[illegible]
different pro[illegible]
from 32 to 3[illegible]
oxygenation [illegible]
Hemoglob[illegible]
tion were m[illegible]
measured be[illegible]

Results

The mean values and extreme ranges of the hemoglobin concentration, packed cell volume, carbon monoxide hemoglobin concentration and erythrocyte 2,3-DPG concentration were 14 7 g per 100 ml (12 0—16 9), 43 % (36—49), 3 1 % (0 8—5 3) and 4 4 mmol per litre of packed red cells (3 7—5 1) respectively There was no significant difference between the 2,3-DPG concentrations before and after the run

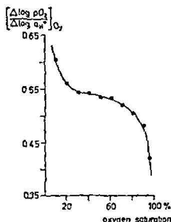


Fig 1

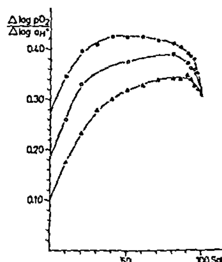


Fig 2

Fig 1 Mean values of the classical Bohr coefficient i e $[\Delta \log pO_2/\Delta \log a_{H^+}]$ at constant (—5 mM) base excess at different oxygen saturation values The pCO_2 difference was 21 to 83 mm Hg and the plasma pH was 7 45 to 7 15

Fig 2 Mean values of the coefficient $[\Delta \log pO_2/\Delta \log a_{H^+}]_{\log pCO_2}$ where log a_{H^+} refers to plasma at different values of the oxygen saturation The different symbols refer to experiments with different pCO_2 ● = 22 mm Hg ○ = 34 mm Hg and Δ = 83 mm Hg The plasma pH interval was 7 15 to 7 45 in the experiments ● and Δ and 7 20—7 40 in the experiments ○

The mean values of the classical Bohr coefficient are shown in Fig 1 The coefficient of variation between the 7 samples varied between 6 and 10 % with the high value at the lower end of the saturation

The mean value of the coefficient $(\Delta \log pO_2/\Delta \log a_{H^+})$ at constant pCO_2 at the three different pCO_2 tensions are shown in Fig 2 The relation between this coefficient and log pCO_2 was found to be approximately linear at all saturation levels The average values over the whole saturation range were 0 29, 0 35 and 0 39 at pCO_2 = 83, 34 and 22 mm Hg respectively The coefficient of variation between bloods in these experiments also ranged from 6 to 10 % with the high value at the lower end of the saturation degree A considerable part of this variation must have been due to experimental error since this is between 5 and 10 % for $\Delta \log pO_2$ and about 5 % for $\Delta \log a_{H^+}$

The mean value of pO_2 at 50 % saturation and a pCO_2 of 34 mm Hg corrected to a plasma pH of 7 30 and 7 40 was 28 6 (S D = 1 3) and 26 2 (S D = 1 2) mm Hg respectively

The mean values of the quotient $(\Delta \log pO_2/\Delta \log pCO_2)$ at constant a_{H^+}, as calculated from the mean values of the experiments at 22 and 83 mm Hg CO_2 tensions, are shown in Fig 3 The mean values over the whole saturation range were 0 12 and 0 08 at the plasma pH values 7 40 and 7 20 respectively

TABLE I Contributions to the classical Bohr effect of protons, carbon dioxide and 2,3-DPG (see text)

% saturation	$[\Delta \log pO_2/\Delta \log a_{H^+}]$	$[\Delta \log pO_2/\Delta \log a_{H^+}]_{\log pCO_2}$	$[\Delta \log pO_2/\Delta \log pCO_2]_{\log a_{H^+}} \times d\log pCO_2/d\log a_{H^+}$	$[\delta \log pO_2/d\log a_{DPG}]_{\log a_{H^+}, \log pCO_2} \times d\log a_{DPG}/d\log a_{H^+}$
10	0.61	0.21	0.30	0.10
20	0.56	0.29	0.22	0.05
30	0.55	0.32	0.18	0.05
40	0.54	0.34	0.16	0.04
50	0.53	0.35	0.15	0.03
60	0.53	0.36	0.13	0.04
70	0.52	0.36	0.12	0.03
80	0.50	0.36	0.11	0.03
90	0.48	0.35	0.10	0.03
95	0.42	0.33	0.08	0.01

Discussion

Previous data

The mean value of 0.53 for the classical Bohr coefficient, i.e. $\Delta \log pO_2/\Delta \log a_{H^+}$ at 50 % saturation and a pH interval of 7.15 to 7.45 in the plasma and with the restriction that the change in log a_{H^+} is accomplished solely and completely by a change in pCO_2, is in good agreement with the range of 0.50—0.54 as obtained from the literature (Hilpert *et al.* 1963, Naeraa *et al.* 1966, Lenfant *et al.* 1969). The mean value of 0.38 of the coefficient $(\Delta \log pO_2/\Delta \log a_{H^+})_{pCO_2}$ at 60 % saturation in a plasma pH interval of 7.20—7.40 and a pCO_2 value of 34 mm Hg is in very good agreement with the single value of 0.38 as calculated from the data of Naeraa *et al.* (1966) at that saturation level, a pH interval of 7.1—7.5 and a constant pCO_2 of 40 mm Hg.

The classical Bohr effect

On the basis of the present data we can estimate the contribution of all the three ligands to the classical Bohr effect. An example of a calculation where the pH is changed, in normal blood, by an increase in pCO_2 from 22 to 83 mm Hg is shown in Table I. Here, the coefficient $(\Delta \log pO_2/\Delta \log a_{H^-})_{\bar{O}}$, is that determined directly (*i.e.* the data in Fig. 1). The coefficient $(\Delta \log pO_2/\Delta \log a_{H^+})_{\bar{O}, pCO_2}$ is that calculated at $pCO_2 = 52$ mm Hg from the data in Fig. 2. The product of the 2 factors $(\Delta \log pO_2/\Delta \log pCO_2)_{\bar{O}, a_{H^+}}$ and $(\Delta \log pCO_2/\Delta \log a_{H^+})$ is that obtained directly from the data in Fig. 3 and the corresponding measured values of $(\Delta \log pCO_2/\Delta \log a_H$. The difference between the first coefficient and the sum of the second coefficient and the product is, according to eq. (7), equal to the product of the following two factors $-[\partial \log pO_2/\partial \log a_{DPG}]$ at constant pCO_2 and a_{H^+} and $(\Delta \log a_{DPG}\ \Delta \log a_{H^+})$, it is found to vary between 0.01 and 0.10 in the saturation range of 95 % to 10 %. This value should be compared to the data obtained by direct studies on the binding of 2,3 DPG to hemoglobin (Garby and de Verdier 1971 a and b). The first factor is, by virtue of eq. (12), equal to $-[\partial \overline{DPG}/\partial \bar{O}_2]_{pCO_2}$ ... and is about 0.05 for

$\partial \bar{O}_2 = 1$. The second factor can be evaluated though eq. (8), where the first term is about 0.7 and the second term about 0.07 for $\partial \bar{O}_2 = 1$. The product of the two factors is therefore about 0.03, i.e. similar to the range of 0.01 to 0.10 as obtained from the present study. The fact that the value is lower at high saturation levels is in agreement with the data obtained by Tyuma *et al.* (1971) showing that the effect of 2,3 DPG on the oxygen affinity of hemoglobin, in a system buffered with respect to 2,3 DPG, vanishes at high saturation levels.

Oxygen-linked proton- and carbamino binding

The present data make it possible to draw conclusions with respect to the details of the mechanisms by which protons and carbon dioxide interact with hemoglobin during oxygenation in intact red cells. Before we discuss these mechanisms, however, it is necessary to examine to what extent the present data on proton and carbamino-linked oxygen affinity can be translated into oxygen linked proton binding and oxygen linked carbamino binding. This will be made by estimating, from the present data and from studies on the binding of 2,3 DPG to hemoglobin (Garby and de Verdier 1971 a and b), the numerical values of the quotients $[\partial \log pO_2/\partial \log a_{H^+}]$ (at constant $\bar{O}_2$, pCO_2 and a_{DPG}) and $[\partial \log pO_2/\partial \log pCO_2]$ (at constant $\bar{O}_2$, a_{H^+} and a_{DPG}) at different oxygen saturation levels from eq.s (5) and (6) and by using eq.s (11) and (13).

We consider first eq. (5). The second term on the right hand side can be estimated (Garby and de Verdier 1971 a) to be about 0.05 × 0.66 = 0.03 as a mean value over the whole saturation range. This term is small compared to $(\Delta \log pO_2/\Delta \log a_{H^+})_{\bar{O}_2}$ and we can conclude that the data in Fig. 2 represent fairly accurately the oxygen linked proton binding at constant DPG activity. Numerically, the oxygen linked proton binding is obtained from Fig. 2 by dividing the values by 0.79 to account for the differences in pH changes in plasma and red cells (Siggaard-Andersen and Salling 1971).

We next consider eq. (6). The second term on the right hand side can likewise be estimated from binding studies (Garby and de Verdier 1971 b) and is about 0.05 × 0.047 = 0.002. This figure is small compared to the values of $(\Delta \log pO_2/\Delta \log pCO_2)_{\bar{O}_2, a_{H^+}}$ and we can conclude that the data in Fig. 3 are good estimated of the oxygen linked carbamino binding.

We now examine our estimates of the oxygen linked proton binding in the light of the very extensive studies made by Siggaard Andersen (1971) and Siggaard-Andersen and Salling (1971). In their experiments the number of protons liberated per heme during oxygenation at constant pCO_2 and constant pH was determined by titration of erythrolysates and whole blood. The relation of their data to the present data can be expressed through eq. (10) when $d\bar{O}_2 = \Delta\bar{O}_2 = 1$. We proceed then to estimate $[\partial \bar{H}^+/\partial \bar{O}_2]_{a_H, a_{DPG}, pCO_2}$ by considering the second term on the right hand side of eq. (10). The value of the 2 factors can be estimated from binding studies through eq. (14) and are about 0.3 and 0.03 respectively. The value of $\Delta\bar{H}^+/\Delta\bar{O}_2$ when $\Delta\bar{O}_2 = 1$ at a plasma pH of 7.30 and a pCO_2 of 34 mm Hg for

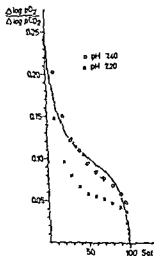


Fig 3

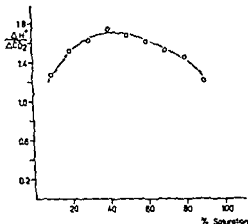


Fig 4

Fig 4 Carbamino-linked proton formation in normal human blood The data are calculated from the data in Fig 2 and 3

whole blood with a normal concentration of 2,3 -DPG was found to be —0 36 by Siggaard Andersen and Salling (1971) so that the estimate of $[\partial\bar{H}^{+}/\partial\bar{O}_2]$ at constant values for a_{H^+}, pCO_2 and a_{DPG} becomes —0 37 This figure should be compared to the estimate of —0 35 of the average value of the data at $pCO_2 = 34$ mm Hg in Fig 2 At $pCO_2 = 22$ mm Hg and 83 mm Hg, the present estimates are —0 39 and —0 28 respectively, as compared to the values of —0 39 and —0 28 as obtained from the data of Siggaard Andersen and Salling (1971) The agreement is well within the experimental error of the present method Recently, Siggaard Andersen *et al* (1971) have confirmed that the difference between the Haldane coefficient, measured as described above, and the Bohr coefficient, measured at constant pCO_2 and at 50 % saturation, on the same blood is very similar indeed to the differences found in Fig 2 between the value at 50 % saturation and the average value over the whole saturation range

Ligand interaction during oxygenation

The present data have implications on the ideas that have emerged during recent years concerning the biochemical and molecular events that take place when the hemoglobin molecule becomes oxygenated Before discussing these implications it is necessary to review briefly the present concepts According to the model of Perutz

(1970), the quaternary structure of the deoxy-conformation of the molecule is stabilized by a number of specified salt bridges between and within the subunits The successive binding of oxygen molecules induces conformational changes in the tertiary structure of the subunits so that the salt bridges between and within them are broken These successive changes result in an increased oxygen affinity of the new species formed at each step At a certain stage of the oxygenation, the molecule flips over into a new quaternary structure, i e the oxy conformation This change is accompanied by a release of the 2,3 DPG molecule and probably occurs when the second molecule of oxygen has been bound

Four of the salt bridges, i e valine NA1 (α_1 and α_2) to arginine HC3 (α_2 and α_1) and histidine HC3 (β_1 and β_2) to aspartate FG1 (β_1 and β_2), involve protons bound to nitrogen When the bridges are ruptured, the pK values are lowered so that these protons are released Protons are also released during oxygenation from histidine H5α

Carbon dioxide can react with the NH_2 groups of all the 4 N terminal valines to form carbamino residues In horse hemoglobin, all the 4 valines exhibit oxygen linked carbamino binding at physiological pH and in the absence of 2,3 DPG (Kilmartin and Rossi Bernardi 1969) The reason for the higher affinity of CO_2 to deoxyhemoglobin is not clear but probably involves an oxygen dependant linkage between the carbamino group to positively charged residues in the sister chain Formation of a carbamino group is accompanied by the release of at least one proton so that the oxygenation leads to a consumption of protons by these reactions

The present data show that the oxygen linked carbamino formation is highly dependent on the degree of oxygen saturation In order to clarify further at which oxygenation steps the reaction occurs, the following analysis was performed The relative number of transitions between the different species i e Hb Hb(O_2), Hb(O_2)$_2$ Hb(O_2)$_3$ and Hb O_2)$_4$ during a small saturation interval over the whole saturation range, was calculated by using Adair s model (1925) and the constants for whole blood at pH 7 40 and pCO_2 = 40 mm Hg given by Roughton *et al* (1971) Values for the oxygen linked carbamino formation i e $\Delta\overline{CO}_2/\Delta\overline{O}_2$ were then given to each of the 4 transitions and the results compared with the experimental data in Fig 3 The 2 curves in the figure correspond to the models where only the first and the 2 first transitions respectively i e Hb→Hb O_2 and Hb(O_2 →Hb(O_2)$_2$ are accompanied by carbamino formation 0 5 and 0 25 mol mol of O_2 respectively Significant values of $\Delta\overline{CO}_2$ $\Delta\overline{O}_2$ for the third and fourth transitions give results that are incompatible with the data From these results 2 conclusions seem warranted 1) that the reaction takes place mainly when the first 2 possibly only the first one) oxygen molecules bind and 2) that under physiological conditions only 2 (possibly only one) of the N terminal valines are involved in carbamino binding The second conclusion is not rigorous since the Adair model only allows for the existence of 5 different species, whereas the existence of mixed species e g Hb(O_2)$_2$ with both oxygens on the α chains and Hb O_2)$_2$ with one oxygen on an α chain and one on a β chain, cannot at present be excluded

In the deoxygenated conformation, the ligand pocket of the α chain is wider that that of the β chain (Perutz 1970) Furthermore, reduction of acid methemoglobin crystals with ferrous citrate or sodium dithionite *shows that ligands are removed* from the β chains more easily than from the α chains (Perutz 1970) It is therefore reasonable, but not necessary, to assume that the α chains are the first to become *oxygenated It follows that it is the N terminals of the α chains that are mainly* involved in the oxygen linked carbamino formation under physiological conditions The difference between the present result and the results of *Kilmartin and Rossi-*Bernardi (1969, see above) may be the presence of 2,3 DPG, since this compound has been shown to inhibit the effect of CO_2 on the oxygen affinity both in dilute *hemoglobin solutions (Tomita and Riggs 1971) and in erythrolysates* (Siggaard-Andersen *et al* 1972)

The present data concerning the effect of carbon dioxide on the oxygen linked proton binding and on the oxygen linked carbamino binding (Fig 2 and 3) allow calculations of the number of protons that are consumed per molecule of CO_2 *released during oxygenation* The result is shown in *Fig 4 If it is assumed that all* these protons originate from the reactions



$$RNH_3^+ \rightleftarrows RNH_2 + H^+$$



$$RNH_2 + CO_2 \rightleftarrows RNHCOO^- + H^+$$

the *theoretical value of the quotient $\Delta\bar{H}^+/\Delta\overline{CO}_2$ should be between 1 and 2* (Rossi Bernardi and Roughton 1967) The present data, showing a value of 1 3—1 7, depending on the oxygen saturation level, are in good agreement with this idea about the origin of the protons The value of K_z, *i e* the ionization constant of the first reaction, can be calculated from the formula of Rossi Bernardi and Roughton (1967)

$$\Delta\bar{H}^+/\Delta\overline{CO}_2 = (K_z + 2\,[H^+])/(K_z + [H^+])$$

and is found *to be 0 3—1 3 $\times 10^{-7}$* mol l

The relative contribution of the 3 pairs of residues that take part in the proton exchange during oxygenation under physiological conditions cannot be exactly evaluated at the present time, mainly because of lack *of knowledge of the order of* oxygen binding to the different chains If following Perutz (1970) we assume that the α chains are the first to become oxygenated and that mixed species are relatively unimportant the following picture emerges Upon binding of the first 2 oxygen molecules to the α chains protons are released from the histidines H5 α and from the non liganded valines NA1 α This proton release is about 0 8 equivalents per mole of oxygen At the same time, 1 3 to 1 7 protons are consumed in the reaction where one molecule of CO_2 is *released from the liganded valines NA1 α* The net result is a proton release which increases (per molecule of O_2) up to about 50 to 60 % oxygen saturation When the β chains become oxygenated, protons are released from the histidines HC3 β, this however, is much smaller about 0 4 per oxygen molecule

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Convergence of Large Muscle Spindle (Ia) Afferents at Interneuronal Level in the Reciprocal Ia Inhibitory Pathway to Motoneurones

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Abstract

Hultborn, H and M Udo *Convergence of large muscle spindle (Ia) afferents at interneuronal level in the reciprocal Ia inhibitory pathway to motoneurones* Acta physiol scand 1972 84 493—499

It has earlier been established that in a number of motor nuclei Ia inhibition can be evoked from [illegible] this convergence takes place already at [illegible] the different muscles is exerted through [illegible] t motoneuronal level With intracellular [illegible] from motoneurones and utilizing the technique of spatial facilitation it has been demonstrated that when activity in Ia afferents from several muscles can evoke inhibition in a given motoneurone a convergence of these Ia afferents does occur onto common interneurones

Lloyd (1941, 1946) originally proposed that the reciprocal inhibition of motoneurones from large muscle spindle (Ia) afferents is exerted monosynaptically, hence the term 'direct inhibition' Later studies showed, however, that an interneurone must be interpolated in the Ia inhibitory pathway (Eccles Fatt and Landgren 1956, R M Eccles and Lundberg 1958 a, Araki, Eccles and Ito 1960, Eide, Lundberg and Voorhoeve 1961) With the technique of spatial facilitation R M Eccles and Lundberg (1958 a) revealed that Ia afferents in different nerve branches from a single muscle converge onto common Ia inhibitory interneurones, thus also demonstrating an appreciable subliminal fringe in this interneuronal pool For most species of hindlimb motoneurones it was found (R M Eccles and Lundberg 1958 b) that volleys in Ia afferents from several muscles may evoke Ia inhibition Consequently they brought up the possibility that this wide spread convergence could take place already at the interneuronal level The alternative would of course be that the Ia inhibition from the different muscles is exerted through separate interneuronal pools which then converge at motoneuronal level This problem has now been analyzed as a part

of an extensive study concerning facilitatory and inhibitory convergence in the Ia inhibitory pathway (cf Lundberg 1970 Hultborn, Jankowska and Lindstrom 1971 a b c, Hultborn and Udo 1972) In this study it will be shown that a convergence of Ia afferents from different muscles does occur onto common inhibitory interneurones

Methods

Most of the present material was collected from the same experiments which were presented in a preceding paper in which also the experimental procedures were described (Hultborn and Udo 1972) Intracellular recording was made from α motoneurones in L5-S1 innervating different hindlimb muscles Since the ventral roots were sectioned the identification of the motoneurones by their antidromic invasion was impossible and they were instead classified according to their Ia receptiveness (Eccles Eccles and Lundberg 1957 R. M Eccles and Lundberg 1958 b) Two additional experiments especially devoted to this project, were performed on spinalized unanesthetized anemically decorticated cats (Anden et al 1966) In these two cats the ventral roots were left intact in order to allow identification of motoneurones by their antidromic invasion No significant difference could be detected between the different preparations besides that the subliminal fringe in the Ia inhibitory interneuronal pools tended to be more pronounced in the anesthetized (chloralose or light Nembutal anesthesia, see Hultborn and Udo 1972) than in the unanesthetized preparations

Abbreviations EPSP excitatory postsynaptic potential IPSP inhibitory postsynaptic potential Q quadriceps VCr vasto-crureus R rectus femoris Sart sartorius Add adductor femoris and longus Grac gracilis PBSt posterior biceps and semitendinosus PB posterior biceps St semitendinosus ABSm anterior biceps and semimembranosus AB anterior biceps Sm semimembranosus G S gastrocnemius and soleus LG Sol lateral gastrocnemius and soleus MG medial gastrocnemius Pl plantaris FDL flexor digitorum and hallucis longus Per the whole peroneal nerve

Results

Spatial facilitation has been used to investigate the occurrence of excitatory convergence of Ia fibres from different muscles onto inhibitory interneurones in the disynaptic Ia inhibitory pathway to antagonist motoneurones Totally 123 motoneurones belonging to different hindlimb muscles were recorded and the results derived from them have been summarized in Table I

In each motoneurone the pattern of Ia inhibition was first determined Subsequently all nerves in which Ia volleys evoked a disynaptic IPSP were tested in pairs Since the distance to the spinal cord from the stimulation sites differs from one nerve to another the precise timing of the stimulus shocks to the nerves tested together was adjusted in order to allow their Ia volleys to arrive simultaneously to the spinal cord Weak stimulus strengths were used so that no or almost no IPSPs were evoked on separate stimulation of single nerves If conjoint stimulation of two nerves produced an IPSP larger than the sum of the two IPSPs caused by separate stimulation this proved the existence of a convergence conclusive in Table I A failure to demonstrate a spatial facilitation (inconclusive in Table I) does however, not necessarily invalidate the possibility of a convergence of Ia afferents onto common interneurones—the failure can e g be explained by a small subliminal fringe which would tend to keep the occlusion (also a consequence of convergence) as large as or even larger than the facilitation

TABLE I *Convergence of excitation from Ia afferents from different muscles on common interneurones in the Ia inhibitory pathway* The species of motoneurone investigated are indicated to the left and the figures in the parentheses indicate the number of cells tested Nerve combinations tested for spatial facilitation are given in horizontal rows separately for each species of motoneurone The results are divided into two classes "conclusive" when a spatial facilitation was established (which thus proved a convergence) and "inconclusive" when the efforts to show the spatial facilitation failed (see further in text) The figures presented for each nerve combination show the numbers of motoneurones in which "conclusive" results were obtained of the total number of motoneurones tested for that nerve combination

	Motoneurones tested	Nerves tested for convergence					
Sart (13)	conclusive total	V Cr Add 8 8	V Cr Sm 10 11	V Cr AB 6 8	Add Sm 9 9	Add AB 8 8	Sm AB 10 10
V Cr (22)	conclusive total	PB-St 18/21	St Grac 9 13	PB-Grac 6 8			
R (13	conclusive total	PB-St 8 12	St Grac 4 6	PB-Grac 4 5			
Add (8)	conclusive total	R Sart 6/7					
Sm (9)	conclusive total	R Sart 7/8	V Cr Sart 3 5				
AB (9	conclusive total	R Sart 1/1	Q Sart 5 8				
PBSt (20)	conclusive total	V Cr R 9 10	R-Sart 1/2	V Cr Sart 3 4	Q-Sart 8 8		
Grac (3)	conclusive total	V Cr R 3 3					
Per (26)	conclusive/total	MG-LG 14 17	Pl FDL 9 10	G-S-Pl 11/16	G-S-FDL 2 3		

Typical findings in motoneurones belonging to the hip flexor Sart are illustrated in Fig 1 Separate stimulation of the nerves to hip extensor muscles Add, Sm and AB and knee extensor V Cr evoked Ia IPSPs which is in accordance with the results of R. M Eccles and Lundberg (1958 b) In records A—F it is shown that spatial facilitation occurred in all possible combinations for these nerves Special attention should be paid to the finding that the convergence onto common interneurones does occur not only from Ia afferents from the different hip extensor muscles (D—F) but also from the knee extensor V Cr and the various hip extensor muscles (A—C) The results from the 13 Sart motoneurones investigated are summarized in the uppermost part of Table I and it emerges that a spatial facilitation in all these combinations was a quite regular finding

Motoneurones innervating V Cr and R receive Ia inhibition from the knee flexors PB St and Grac though the effect from the latter is often weak R. M Eccles and Lundberg 1958 b) The convergence of Ia afferents from these knee flexors on interneurones in the inhibitory pathway to V Cr motoneurones is illustrated in Fig 2 Records in A reveal an evident spatial facilitation on conjoint stimulation of PB and

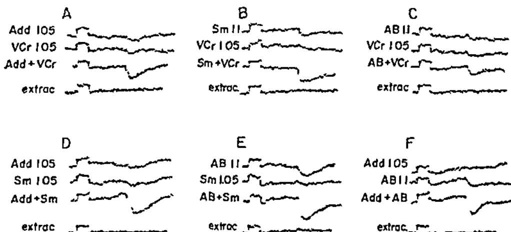
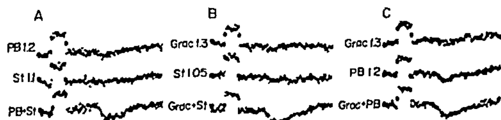


Fig 1 *Excitatory convergence of Ia afferents on interneurones inhibiting a Sart motoneurone* The three upper traces in all records (A—F) are averaged intracellular responses (positivity signalled upwards in this and following figures) The lowermost trace is recorded with the microelectrode withdrawn to a just extracellular position and show the lack of any field potentials to the combined nerve stimulation (as for the third trace) The calibration pulses in the beginning of all traces have an amplitude of 0 5 mV and a duration of 2 ms

St nerves In records B and C a similar facilitation appears after conjoint stimulation of Grac and St and of Grac and PB respectively From the material presented Table I it can be seen that a convergence of Ia afferents from the different knee flexor muscles does occur on interneurones inhibiting VCr as well as R motoneurones

Motoneurones to hip extensors AB Sm and Add receive Ia inhibition from their antagonists iliopsoas and Sart as well as R (the latter giving only very weak inhibition in Add motoneurones) Some motoneurones belonging to AB and Sm muscles also draw their Ia inhibition from the "unrelated" pure knee extensor VCr (R. M Eccles and Lundberg 1958 b) Of these nerves only those to Sart, R and VCr (the two latter often dissected together) have been used for stimulation during the present investigation From Table I it appears that a facilitation occurs in the Ia inhibitory pathway to hip extensor motoneurones on conjoint stimulation of R and



a VCr motoneurone ... the beginning of the

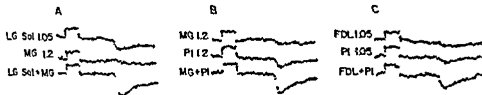


Fig 3 *Excitatory convergence of Ia afferents on interneurones inhibiting Per motoneurones* All traces are averaged intracellular responses Records in A—B are from one Per motoneurone while the records in C are from another Per motoneurone The calibration pulses in the beginning of all traces in A—C have an amplitude of 0 5 mV and a duration of 2 ms

Sart and also of VCr and Sart (the latter combination tested only in Sm motoneurones)

Knee flexor motoneurones St PB and Grac receive strong inhibition from VCr and R and a subsidiary inhibition in St motoneurones is also evoked from Sart (R. M Eccles and Lundberg 1958 b) In the present material also 2 PB motoneurones (defined as belonging to PB by larger max Ia EPSP from PB than from St as well as lack of Ia EPSP from ABSm cf Eccles Eccles and Lundberg 1957) disclosed minute Ia IPSPs on stimulation of the Sart nerve Again it was revealed (Table I) that convergence of Ia afferents from different muscles onto common interneurones occurs

As described above Ia afferents from ABSm and VCr converge onto the inhibitory interneurones which impinge on Sart motoneurones Are the same VCr coupled interneurones also inhibiting knee flexor motoneurones? If so one would expect at least small occasional Ia IPSPs in PBSt motoneurones from ABSm but since such IPSPs are consistently lacking (R M Eccles and Lundberg 1958 b) it is very unlikely that the same VCr coupled interneurones inhibit both Sart and PBSt motoneurones We have nevertheless tried to facilitate small Ia IPSPs evoked from VCr in 9 PBSt motoneurones by maximal Ia volleys in the ABSm nerve No significant facilitation could be seen at any occasion Interneurones mediating reciprocal Ia inhibition which receive an excitatory convergence from Ia afferents from VCr and ABSm seem thus not to project to the PBSt motor nucleus

In motoneurones supplying the common peroneal nerve the Ia inhibition is evoked from MG LG Sol Pl and FDL (although the inhibition from some of these nerves may be lacking in individual motoneurones) As exemplified in Fig 3 and shown in Table I the results conform to the general pattern described i e that whenever stimulation of more than one nerve evokes a Ia IPSP it is usually possible to demonstrate that a convergence takes place already at the interneuronal level

Discussion

Widespread convergence of Ia inhibitory action on a number of motor nuclei was found by R. M Eccles and Lundberg (1958 b) and they brought up the possibility that this convergence could occur already at the interneuronal level With the technique of spatial facilitation of Ia IPSPs we have now demonstrated that when

ever activity in Ia afferents from several muscles evokes inhibition in a given motoneurone a convergence of these Ia afferents does occur onto common interneurones

These conclusions are thus in full agreement with the recent findings of a similar convergence of Ia afferents on direct recording of interneurones (Hultborn *et al* 1971 b, Hultborn and Santini to be published) which were strongly supposed to mediate the reciprocal Ia inhibition to motoneurones (Hultborn *et al* 1971 b) In fact interneurones identified with those criteria were recently shown to produce monosynaptic IPSPs in motoneurones (Jankowska and Roberts 1971)

Impulses in Ia afferents in a given nerve may often evoke Ia inhibition in several motor nuclei The question can then arise whether individual Ia inhibitory interneurones excited from such a nerve evoke inhibition in motoneurones of all the concerned motor nuclei or whether subgroups of interneurones exist each of them inhibiting only certain species of motoneurones The present results favour the last alternative and indicate that the destination of individual Ia inhibitory interneurones excited by this nerve may often be deduced by its pattern of Ia excitatory convergence For example Ia inhibitory interneurones with a convergence from VCr and ABSm should impinge hip flexor motoneurones while those interneurones with a convergence from VCr and Sart should be expected to inhibit knee flexor (PBSt) or hip extensor (ABSm) motoneurones

Muscles acting as close synergists exhibit a high degree of excitatory monosynaptic interconnexions but some muscles of more unrelated function may also be connected by such monosynaptic actions Often although not always the pattern of convergence of Ia excitation of motoneurones and the pattern of convergence of reciprocal Ia inhibition is similar For example close synergists as PB and St which always exhibit a strong monosynaptic interconnexion also both evoke Ia inhibition in Q motoneurones A more complex pattern can be exemplified by the excitatory convergence between Ia afferents from knee extensors (VCr) and hip extensors in VCr and some ABSm motoneurones Correspondingly both the hip extensors and the knee extensor VCr give rise to reciprocal inhibition of hip flexor motoneurones The tentative functional importance of the convergence of hip and knee extensor Ia afferents onto hip extensor motoneurones during the stance phase of the step has been discussed by Engberg and Lundberg (1969) and Lundberg (1969) It can be assumed that the discharge of the hip extensor motoneurones often depends on the spatial facilitation resulting from simultaneous activity in the hip and knee extensor Ia afferents Engberg and Lundberg (1969) and Lundberg (1969) suggested that the Ia effects from knee muscles may give a high safety factor for the firing of hip extensor motoneurones and thus to the hip extension which is of paramount importance for the forward propulsion in locomotion They also considered the convergence of Ia inhibition from hip extensors and knee extensor VCr onto hip flexor motoneurones in similar terms and the question then arises if also the Ia inhibition of hip flexor motoneurones may be dependent on a conjoint activity in hip and knee extensor Ia afferents In order to achieve the necessary spatial facilitation an excitatory convergence must take place somewhere and in the disynaptic Ia inhibitory pathway

this can obviously only be obtained by a convergence at the interposed interneurones The present findings of a convergence corresponding to that of Ia afferents on motoneurones thus points to a close resemblance in the mode of excitatory control by Ia afferents of Ia inhibitory interneurones and motoneurones

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Bretylium and the Supersensitivity to Exogenous Noradrenaline after Sympathetic Denervation Studied in the Conscious Rat

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Abstract

LUNDBERG, D *Bretylium and the supersensitivity to exogenous noradrenaline after sympathetic denervation studied in the conscious rat* Acta physiol scand 1972 84 500—505

Rats denervated by excision of the superior cervical sympathetic ganglion and given reserpine at the time of operation were used The width of the palpebral aperture which reflects the tone of the sympathetically innervated periorbital smooth muscle was measured in the conscious rats after different *i p* doses of l noradrenaline The sensitivity of the muscle to exogenous noradrenaline was thus tested during 3 periods of 3 h duration each between 13 and 30 h after denervation Bretylium which is known to delay the degeneration contraction of the muscle *i e* the degeneration transmitter release postponed the development of denervation supersensitivity Hence bretylium seems to preserve temporarily both the amine storage mechanisms and the axonal amine pump in the degenerating adrenergic nerve terminals

Axotomy of postganglionic sympathetic nerves starts a series of events in the denervated organ which is due to the degeneration and happens during the first few days after the denervation Some of these effects have recently been extensively studied (for ref, see Pluchino *et al* 1970 and Lundberg 1970 a) Bretylium and some related quaternary ammonium compounds delay the onset of the degeneration loss of sympathetic transmitter (rat Benmiloud and Euler 1963 Malmfors and Sachs 1965, cat Pluchino *et al* 1970) and some degeneration stimulation effects due to leakage of transmitter from the degenerating nerve terminals (rat Lundberg 1969 and 1970 b, cat Langer 1966 rabbit Trendelenburg and Barany 1970) Recently, it was found by Pluchino *et al* (1970) that β TM 10 certainly delayed the degeneration leakage of sympathetic transmitter from the terminals of the cat nictitating membrane, but it did not alter the developments of other consequences of degeneration such as supersensitivity to exogenous noradrenaline and signs of degeneration in electron micrographs

Bretylium is very active in delaying the start of the degeneration contraction of the rat periorbital smooth muscle, whereas bethanidine almost completely lacks this property (Lundberg 1970 b) Otherwise the two drugs have many pharmacological activities in common (see Boura and Green 1965) In the present study it was tested whether bretylium could alter the progress of denervation supersensitivity to noradrenaline in the periorbital smooth muscle of the conscious rat Bethanidine acted as a control substance It was found that bretylium, but not bethanidine, delayed the appearance of denervation supersensitivity A comparison between β TM 10 and bretylium in the rat would have been interesting However, since β TM 10 almost lacks ability to delay the degeneration transmitter release at untoxic dose levels in this species (Lundberg 1969 1970 b) such a comparison seemed difficult to perform.

Materials and Methods

Male Sprague Dawley rats (b wt 250—350 g) were used Under ether anesthesia the right superior cervical ganglion was excised (denervation) and the left preganglionic trunk was cut (decentralization)

Drugs

The [illegible] e and [illegible] basil® [illegible] were [illegible] that of noradrenaline which is calculated as the base

Estimation of the sensitivity to exogenous noradrenaline in vivo

The width of the palpebral aperture which reflects the tone of the sympathetically innervated periorbital smooth muscle was intermittently measured using the method described earlier (for details see Lundberg 1969) In order to eliminate the tone of the muscle due to the spontaneous release of stored transmitter from the degenerating nerve endings (the degeneration contraction) the rats were treated with 2 5 mg/kg of reserpine i p at the time of the denervation This dose of reserpine is known to cause a longlasting and complete disappearance of noradrenaline from sympathetically innervated organs in the rat without interfering with the amine uptake mechanism at the level of the axonal membrane (Malmfors 1965) Each rat was given 3 series of l noradrenaline injections i p in the course of one experiment. Each series consisted of 3 injections of different doses of l noradrenaline (0 1, 0 2 and 0 4 mg/kg or 0 05 0 1 and 0 25 mg/kg) given 1 h apart The series were started at 13 19 and 27 h after denervation respectively The palpebral aperture was measured at least twice just before the start of each injection series and once at 15 30 45 and 60 min after each injection The peak increase in palpebral aperture (usually obtained at 15 min) was used as a measure of the noradrenaline effect The effect had always disappeared at 60 min

Results

3 groups of rats (n = 4) were studied 12 h after the operation the groups were injected s c with bretylium (4 mg/kg) bethanidine (4 mg/kg) or 0 9 % NaCl respectively

Fig 1 shows the maximum widening of the palpebral apertures after the i p noradrenaline injections In the bretylium group the response to noradrenaline was distinctly greater at the first test period 13—16 h after denervation than at 19—22 h on both the denervated and the decentralized (control) side In the bethanidine group the noradrenaline response on the decentralized side was somewhat greate

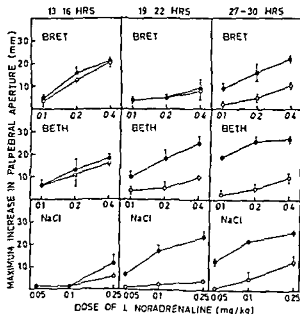


Fig 1 Sensitivity of the periorbital smooth muscle to i p l noradrenaline at different time intervals after denervation The different noradrenaline doses of each test series were given 1 h apart The first dose of the series was given at 13 19 or 27 h respectively The width of the palpebral aperture was measured just before each injection and after 15 30 45 and 60 min The maximum effect of the injection was taken as the noradrenaline effect There were 4 rats in each group M ± S E M are shown Filled circles represent the denervated side open circles the decentralized side

at 13—16 h than at 19—22 h Thus there was an early transient increase in sensitivity after bretylium and possibly after bethanidine The two drugs are known to inhibit monoamine oxidase (Clarke 1970) This effect could be responsible for the early supersensitivity found even if it is not clear why no supersensitivity remained at 19—22 h where the sensitivity in the bretylium group was low on both sides In the bethanidine and the NaCl groups however the sensitivity on the denervated side was distinctly increased compared to that on the decentralized side The magnitude of the denervation supersensitivity was about the same in the two groups At the time of the last noradrenaline test 27—30 h there was a clear cut supersensitivity on the denervated side also in the bretylium group

The noradrenaline tests were performed during the time interval after the denervation when the degeneration contraction of the periorbital smooth muscle normally or after bretylium is known to take place In order to facilitate a comparison of the time course of the development of denervation supersensitivity found in the present experiment with that of the degeneration contraction found earlier Fig 2 was constructed The maximum effects of the different noradrenaline doses on the denervated side in the bretylium and the bethanidine groups are shown in the figure as columns in a three dimensional diagram bretylium black bethanidine white In the 'bottom of the box the times of half maximum contraction on the ascending phase (T_{50a}) and the descending phase (T_{50d}) of the degeneration contraction are indicated (M ± S E M) These values are from Lundberg 1970 a (bretylium) and 1970 b (bethanidine) where the rats were treated as in the present experiment except the reserpine and the noradrenaline injections In these studies the T_{50a} and the T_{50d} after bretylium treatment were very much delayed whereas the correspond

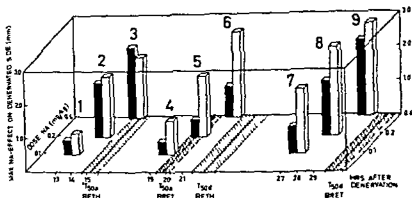


Fig 2 Sensitivity of the periorbital smooth muscle to i p l noradrenaline in relation to earlier findings of the time course of the degeneration contraction (Lundberg 1970 a and b) The

phase (T_{50a}) and the descending phase (T_{50d}) of the degeneration contractions are indicated (M±S E M)

ing values after bethanidine did not significantly differ from those of untreated controls The latter values are therefore not shown in the figure Unfortunately, it is impossible to compare the degree of denervation supersensitivity at around the expected T_{50a} of the present two groups in the figure, because of the drug induced early supersensitivity at the first test period which just precedes the T_{50a} of bethanidine (columns 1—3) It is notable however that around T_{50a} of bretylium the sensitivity in the bretylium group was still quite low (black columns 4—6, see also Fig 1) At the test periods just preceding T_{50d} i e the time interval following the peak of the degeneration contraction the sensitivity was increased and almost equally developed in the bethanidine and the bretylium groups compare the white columns 4—6 with the black ones 7—9 Hence there seems to be quite a good temporal correlation between the course of degeneration contraction and the progress of denervation supersensitivity after both bretylium which delays and bethanidine which does not delay

Discussion

The Trendelenburg group has recently performed a systematic study of the effects of the bretylium like adrenergic neuron blocker β TM 10 on several events induced by sympathetic denervation of the cat nictitating membrane (Pluchino *et al* 1970 They injected β TM 10 at a stage of the degeneration contraction of the nictitating membrane equivalent to our T_{50a} which is the time of half development of the degeneration contraction They found that the drug divided the contraction into two parts and delayed the latter part for several h During the interval of rest between the two contractions the gradual decline in transmitter content of the denervated smooth muscle and the decline in granulated vesicles in electron micro-

graphs of adrenergic nerve terminals were interrupted The drug, however, failed to interfere with the progress of other degeneration events, such as the signs of degeneration in electron micrographs (except the loss of granular vesicles), the decreasing capacity to take up exogenous noradrenaline *in vitro*, and the supersensitivity to exogenous noradrenaline *in vitro* The authors proposed that β TM 10 did not arrest the degeneration process *per se* but preserved for a while the transmitter in the degenerating terminals by its monoamine oxidase inhibiting effect

In the present experiments bretylium was found to delay the development of denervation supersensitivity in the rat periorbital smooth muscle *in vivo* in a similar way as it is known to delay degeneration contraction, *i e* degeneration transmitter release It is well established that the denervation supersensitivity is causally related to the inability of the degenerating nerve terminals to take up sympathomimetic amines (Langer and Trendelenburg 1966 Smith *et al* 1966, Trendelenburg 1969 and Pluchino *et al* 1970) Therefore it seems reasonable to propose that bretylium preserves both the transmitter storage and the function of the axonal amine pump in the degenerating nerve endings

Hence the effects on degenerating adrenergic nerves of bretylium (in the rat) and β-TM 10 (in the cat) seem to differ The adrenergic nerve effector systems of the two species rat and cat used in the present study and in that of Pluchino *et al* (1970) have many similarities However, different experimental conditions in the two studies *e g* times of injection of the delaying drug (in the present study before in that of Pluchino *et al* (1970) during the expected degeneration con) *in vivo* (present experiment) contra *in vitro* experiments, are probably different to allow any closer comparison It may be added that in the rat β TM 10 is a poor delayer of the degeneration contraction and much less efficient than bretylium (Lundberg 1969 and 1970 b) Therefore the decision whether there is a species difference or a real difference between β TM 10 and bretylium will have to rest on experiments on cats

Bethanidine which acted as a control substance lacks the ability to delay the degeneration contraction (Lundberg 1970 b) and it did not delay supersensitivity Since bethanidine is known to inhibit monoamine oxidase as efficiently as bretylium (Clarke 1970) it seems improbable that the effects of bretylium discussed here are related only to inhibition of monoamine oxidase

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Prostaglandin Induced Inhibition of Neurotransmission in the Isolated Guinea Pig Seminal Vesicle

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Abstract

HEDQVIST P *Prostaglandin induced inhibition of neurotransmission in the isolated guinea pig seminal vesicle* Acta physiol scand 1972 *84* 506—511

The contractile response of the guinea pig seminal vesicle to postganglionic nerve stimulation is inhibited by prostaglandin E_1 and E_2 in low doses and potentiated by high doses Prostaglandin E_1 and E_2 consistently increase the contractile response to exogenous noradrenaline The potentiation by high doses of prostaglandin E_1 of the effector response to nerve stimulation is antagonized by SC 19220 inhibitor of prostaglandin action. The effect of prostaglandin E_1 and E_2 on the neuroeffector system of the seminal vesicle may be explained by a dual action inhibition of noradrenaline release from the nerve terminals and potentiation of the effector response to the noradrenaline released An endogenous prostaglandin mediated regula of the smooth muscle response to nerve activity is suggested

Prostaglandins of the E type (PGE$_1$ PGE) have been shown to inhibit the sympathetic neurotransmission in several different tissues and animal species (Hedqvist 1970 a b 1972 Hedqvist and Euler 1972 Hedqvist and Wennmalm 1971, Wennmalm 1971

In the guinea pig vas deferens the contractile response to postganglionic nerve stimulation is inhibited by low doses of PGE$_1$ and PGE$_2$ and potentiated by high On the other hand the contractile response to noradrenaline (NA) is potentiated by low as well as by high doses of the two compounds indicating a dual action on the neuromuscular transmission prejunctional inhibition and postjunctional potentiation (Hedqvist and Euler 1972

In the present communication it is shown that PGE$_1$ and PGE$_2$ operate by the same mechanism on the neuromuscular transmission in the guinea pig seminal vesicle

Methods

15 guinea pigs weighing 600—800 g were used for the study The animals were killed by a blow on the head and the seminal vesicles were dissected free and mounted in a 5 ml organ bath. As incubation medium was used Tyrode solution of the following composition (concentrations in mM $NaCl$ 136 7 KCl 2 7 $CaCl_2$ 1 8 $MgCl_2$ 0 5 $NaHCO_3$ 11 9 NaH_2PO_4

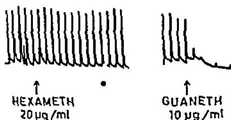


Fig 1 Guinea pig seminal vesicle Recorded contractions in response to postganglionic nerve stimulation (25 pulses, 10/s, 1 ms) resumed each min Hexamethonium (20 µg/ml) and guanethidine (10 µg/ml) added to bath fluid at arrows Washing at dot

0 4 glucose 5.5) The solution was kept at 37° C and was aerated with 6 5 % CO_2 in O_2 The seminal vesicle was stimulated transmurally with biphasic pulses at a frequency of 10/s, a duration of 1 ms and at supramaximal voltage Trains of 25 pulses were given each min All recordings of the mechanical responses of the seminal vesicle preparation were made isotonically by means of a Harvard heart/smooth muscle transducer coupled to a Honeywell Electronik 194 Isotonic registration was considered to be advantageous with regard to the rather large excursions of the preparation By this method a constant load (0 25 g) was maintained throughout the experiment

The following agents were used Guanethidine (Ismelin CIBA), hexamethonium, noradrenaline, prostaglandins E_1 and E_2 1 acetyl 2 (8-chloro-10 11-dihydrodibenz [b f] [1 4] oxazepine-10 carbonyl) (SC 19220 Searle)

Results

Transmural stimulation (25 pulses 1 ms, 10/s) resumed each min caused reproducible contractions of the seminal vesicle preparation The effector responses to this type of stimulation were unaffected by hexamethonium (20 µg/ml) while abolished by guanethidine 10 µg/ml (Fig 1) These observations and the stimulation parameters used indicate that the mechanical response resulted from postganglionic nerve stimulation

Addition to the bath fluid of PGE_1 (1—20 ng/ml) consistently and progressively inhibited the effector response to nerve stimulation (Fig 2) The onset of the inhibition was always rapid and usually manifest within 2—3 min After washing the preparation the contractions rapidly returned to normal height

Administration of a high dose of PGE_1 (100—200 ng/ml) caused a slight contraction of the preparation and potentiated the response to nerve stimulation (Fig 2) In this case a single wash of the preparation was often followed by depressed contractions, which were restored to normal height after repeated washing

SC 19220 (30 µg/ml) claimed to antagonize at least some of the effects of PG s (Sanner 1969 1971), did not alter the inhibitory action of low doses of PGE_1 on the neuromuscular transmission in the seminal vesicle On the other hand, as previously shown in the guinea pig vas deferens (Hedqvist and Euler 1972) SC 19 220 antagonized the potentiation of the responses by high doses (100—200 ng/ml), of PGE_1 leading to inhibition of the responses even in this dose range (Fig 3)

The effect of different doses of PGE_1 on the contractile responses to nerve stimulation is given in Table I

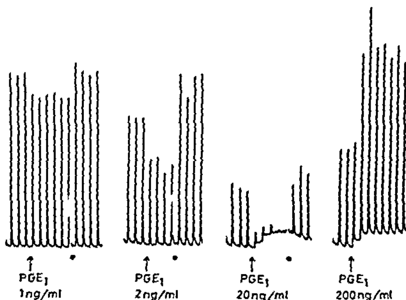


Fig 2 Guinea pig seminal vesicle Recorded contractions to postganglionic nerve stimulation (25 pulses 10/s 1 ms resumed each min PGE_1 added to bath fluid at arrows Washing at dots

When electrical stimulation was replaced by administration of NA (0 6 μg/ml) the ensuing contractions were generally increased by PGE_1 even in doses known to inhibit the responses to nerve stimulation (Fig 4)

PGE produced qualitatively the same effects as PGE_1 on the seminal vesicle preparation although it appeared quantitatively less active Thus low to moderate doses of the compound (2—60 ng/ml) inhibited the responses to nerve stimulation and potentiated the contractile responses to NA (Fig 4 5) In the high dose range (100—200 ng/ml the effect of PGE_2 on the response to nerve stimulation varied from moderate inhibition to slight potentiation However when the dose of PGE_2 was further increased to 1 μg/ml it regularly increased the stimulation response confirming the observations of Naimzada (1969 a b) and Sjostrand and Swedin (1970)

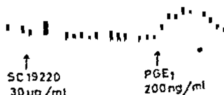


Fig 3 Guinea pig seminal vesicle Recorded contractions to postganglionic nerve stimulation (25 pulses 10/s 1 ms resumed each min SC 19220 (30 μg/ml) and PGE_1 (200 ng/ml) added to bath fluid at arrows Washing at dot

TABLE I Effect of PGE_1 and PGE_1+SC 19 220 on the contractile responses to postganglionic nerve stimulation (25 pulses 10 s 1 ms) in isolated guinea pig seminal vesicles SC 19 220 added to the bath 10 min before PGF, All values are presented as per cent of a control stimulation immediately preceding the addition of PGE_1 and are given as means±S F , n = number of observations

PGE 2 ng ml	n	PGE_1 200 ng/ml	n	PGE_1+SC 19 220 200 ng ml 30 μg ml	n
64±3	6	194±16	4	54±14	4

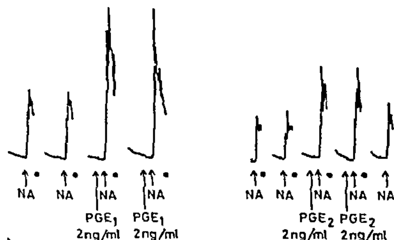


Fig 4 Guinea pig seminal vesicle Recorded contractions to a standard dose of NA (0 6 μg/ml) NA PGE_1 and PGE_2 added to bath fluid at arrows Washing at dots

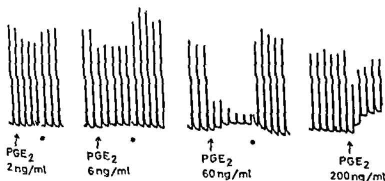


Fig 5 Guinea pig seminal vesicle Recorded contractions to postganglionic nerve stimulation (25 pulses 10/s 1 ms) resumed each min PGE_2 added to bath fluid at arrows Washing at dots

Discussion

PGE_1 and PGE_2 have been shown to potentiate the contractile response to nerve stimulation in the guinea pig seminal vesicle (Naimzada 1969 a b) Sjostrand and Swedin (1970) found the same effect of high doses of the two compounds (0 1—1 0 μg/ml) while lower doses (1—20 ng/ml) left the response unchanged or occasionally caused a slight reduction In the present study it was observed that when PGE_1 and PGE_2 were administered in low concentrations to the medium bathing the seminal vesicle the ensuing contractions to nerve stimulation were markedly inhibited On the other hand high doses of the compounds increased the responses to stimulation The contractile responses to exogenous NA were always potentiated by PGE_1 and PGE_2 even in doses known to inhibit the response to nerve stimulation Inhibition by PGE_1 and PGE_2 of the contractile response to nerve stimulation and potentiation by the same dose of the response to NA indicate different targets By analogy with results in the cat spleen and the rabbit heart (Hedqvist 1970 a, b, Hedqvist and Wennmalm 1971 Wennmalm 1971), it is concluded that the two compounds may act prejunctionally in all probability decreasing the amount of NA released from the nerve terminals in response to nerve activity A dual action may explain the inhibitory action of low doses on the neuromuscular transmission and potentiation by high doses At low doses the two compounds probably act predominantly at the prejunctional level decreasing the release of NA from the nerve terminals At high doses a progressively increasing potentiation of the effector cell response to NA cf Hedqvist and Euler 1972) may mask the prejunctional inhibitory action leading to a net increase of the contractile response to nerve stimulation Further support for a dual action is the observation that SC 19 220 antagonized the potentiation of the contractile response to nerve stimulation by high doses of PGE_1 leading to decreased responses even in this range

It is interesting to note that in the guinea pig vas deferens PGE_1 and PGE_2 have been shown to possess the same type of dual action on the neuromuscular transmission i e inhibition by low doses and potentiation by high doses of the two compounds (Hedqvist and Euler 1972

On the basis of their natural occurrence and availability for release as well as observations on the pharmacological actions of PG s it has been postulated that endogenous PGE_1 and PGE_2 operate on sympathetic neurotransmission by a feed back mechanism and thereby modulate the effector responses to nerve activity (Hedqvist 1970 a b This hypothesis has recently gained in probability by the observation that in the cat spleen and the rabbit heart inhibition of PG synthesis increases the NA release and/or the effector response to nerve stimulation (Hedqvist, Stjärne and Wennmalm 1971 Samuelsson and Wennmalm 1971 Ferreira and Moncada 1971) The inhibitory effect of remarkably low doses of PGE_1 and PGE_2 on the neuromuscular transmission in the guinea pig seminal vesicle observed in the present study, suggests that a similar endogenous PG mediated regulation of the smooth muscle contractile responses to nerve activity may well be present in this tissue.

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Discussion

PGE_1 and PGE_2 have been shown to potentiate the contractile response to nerve stimulation in the guinea pig seminal vesicle (Naimzada 1969 a b). Sjöstrand and Swedin (1970) found the same effect of high doses of the two compounds (0.1—1.0 μg/ml) while lower doses (1—20 ng/ml) left the response unchanged or occasionally caused a slight reduction. In the present study it was observed that when PGE_1 and PGE_2 were administered in low concentrations to the medium bathing the seminal vesicle the ensuing contractions to nerve stimulation were markedly inhibited. On the other hand high doses of the compounds increased the responses to stimulation. The contractile responses to exogenous NA were always potentiated by PGE_1 and PGE_2 even in doses known to inhibit the response to nerve stimulation. Inhibition by PGE_1 and PGE_2 of the contractile response to nerve stimulation and potentiation by the same dose of the response to NA indicate different targets. By analogy with results in the cat spleen and the rabbit heart (Hedqvist 1970 a, b, Hedqvist and Wennmalm 1971, Wennmalm 1971), it is concluded that the two compounds may act prejunctionally, in all probability decreasing the amount of NA released from the nerve terminals in response to nerve activity. A dual action may explain the inhibitory action of low doses on the neuromuscular transmission and potentiation by high doses. At low doses the two compounds probably act predominantly at the prejunctional level, decreasing the release of NA from the nerve terminals. At high doses a progressively increasing potentiation of the effector cell response to NA (cf. Hedqvist and Euler 1972) may mask the prejunctional inhibitory action, leading to a net increase of the contractile response to nerve stimulation. Further support for a dual action is the observation that SC 19220 antagonized the potentiation of the contractile response to nerve stimulation by high doses of PGE_1, leading to decreased responses even in this range.

It is interesting to note that in the guinea pig vas deferens PGE_1 and PGE_2 have been shown to possess the same type of dual action on the neuromuscular transmission, i.e. inhibition by low doses and potentiation by high doses of the two compounds (Hedqvist and Euler 1972).

On the basis of their natural occurrence and availability for release as well as observations on the pharmacological actions of PG's, it has been postulated that endogenous PGE_1 and PGE_2 operate on sympathetic neurotransmission by a feed-back mechanism and thereby modulate the effector responses to nerve activity (Hedqvist 1970 a, b). This hypothesis has recently gained in probability by the observation that in the cat spleen and the rabbit heart inhibition of PG synthesis increases the NA release and/or the effector response to nerve stimulation (Hedqvist, Stjärne and Wennmalm 1971, Samuelsson and Wennmalm 1971, Ferreira and Moncada 1971). The inhibitory effect of remarkably low doses of PGE_1 and PGE_2 on the neuromuscular transmission in the guinea pig seminal vesicle observed in the present study, suggests that a similar endogenous PG mediated regulation of the smooth muscle contractile responses to nerve activity may well be present in this tissue.

and Rubinstein 1966) Likewise certain types of prolonged environmental stress can gradually lead to persistent hypertension in mice (Henry, Meehan and Stephens 1967)

According to recent haemodynamic studies of SHR, systemic resistance is increased in relation to NCR even at complete relaxation of the resistance vessels (Folkow *et al* 1970 a b), in a similar way as earlier shown for the hand and forearm vessels in primary (essential) hypertension in man (Folkow 1956 Conway 1963 Sivertsson 1970) This suggests that the resistance vessels adapt structurally in response to an increased load Further the dose response curve is steeper, at an *unchanged threshold sensitivity*, *and the maximal contractile strength* of the SHR resistance vessels is decidedly enhanced (Folkow *et al* 1970 b) These latter changes strongly suggest the presence of an increased bulk of media which together with the reduced lumen implies a considerable structural increase of the wall/lumen ratio of the average resistance vessel This type of structural vascular adaption to changes in pressure load appears to develop within few weeks (Folkow and Sivertsson 1968 Folkow *et al* 1971 a) and its hemodynamic impact seems to be so *pronounced that it can largely alone explain the raised resistance in the resting* equilibrium both in man and in SHR Therefore these findings hardly support the view that sympathetic activity should be continuously enhanced in primary hypertension neither in man nor in SHR It is on the other hand obvious that the sympathetic nervous system may nevertheless raise the average pressure load by means of an intermittently enhanced activity in the course of daily life and in such a way act as a functional trigger mechanism that precipitates the mentioned type of *per se* normal structural adaptation of the vessels It seems clear from recent studies of the Okamoto group (Okamoto 1969) that the sympathetic nervous system constitutes one of the hereditary elements responsible for the high blood pressure in SHR though also other genetic elements appear to contribute (Louis *et al* 1969)

As earlier discussed with respect to essential hypertension in man (Folkow Grimby and Thulesius 1958 Sivertsson 1970) one such genetic element might be constituted simply by a moderately enhanced tendency of mesenchymal tissues to respond with hypertrophy to a given load One possible way to explore whether such a non neurogenic factor really participates would be to exclude or grossly reduce from early life the impact of the sympathetic nervous system in both SHR and NCR and then to explore their vascular hemodynamics with respect to structurally determined differences In the present study immunosympathectomy was used in an attempt to eliminate as far as possible sympathetic cardiovascular control in newborn SHR and NCR The *intention was to follow their blood pressure in* comparison with sham treated SHR NCR for about one year To explore whether any structural vascular changes were present as a result of the altered neurogenic "pressure load detailed hemodynamic analyses of their resistance vessels were then performed A brief preliminary report has earlier been presented (Folkow *et al* 1971 b)

Methods

12 newborn spontaneously hypertensive rats (SHR) of the Wistar strain (Okamoto 1963) and 8 newborn normal control rats (NCR) were treated with a sympathetic nerve growth factor antiserum (Wellcome Reagents Ltd[1]) The rats, below denoted SHR_{is} and NCR_{is}, were injected for the first 5 days of life with doses which increased as follows 0 1 ml 0 1 ml, 0 2 ml 0 2 ml and 0 4 ml (Zaimis 1967) 14 newborn SHR and 12 newborn NCR were injected with 0 9 % NaCl solution in the same manner Once monthly for the first 8 months blood pressure was recorded in all the rats using a tail colorimetric method This method was employed for the following reasons Because of the *immunosympathectomy, indirect heating could not be* used in SHR_{is} and NCR_{is} for producing tail vasodilatation since this is mainly a result of inhibition of constrictor fibre tone (In fact these animals proved to be very sensitive to heating and could evidently not use their tail or other parts of their cutaneous vascular bed, for thermoregulatory purposes providing good evidence of an effective destruction of adrenergic vasoconstrictor fibres) Instead, the tail was only locally heated in a double wall metal tube where 43° C water was circulating After about 15 min af local heating, when the tail had become pink because of superficial vasodilatation it was emptied of blood, and hence blanched by application of a suprasystolic pressure inside a plexiglass cylinder surrounding the tail Then a cuff placed around the base of the tail was inflated to suprasystolic pressure whereupon the blanched tail was taken out of the plexiglass cylinder The cuff pressure was slowly decreased while the colour of the tail was observed The pressure at which the first signs of a skin flush appeared was defined as the systolic blood pressure Even though this method is rather inexact it made it possible to follow the development of the "resting" blood pressure level fairly well also in the immunosympathectomized rats The conventional plethysmographic method proved to be less reliable when employed in these animals, since it calls for a substantial widening also of the deeper resistance vessels of the tail which in normal animals occurs as a result of reflex sympathetic inhibition when they are indirectly heated Direct heating presumably dilates mainly the most superficial small vessels which determine skin colour but may far less affect the main resistance vessels of the tail Thus, tail blood flow does not appear to be enough increased by local heating alone to make the plethysmographic method fully reliable while the superficial dilatation allows for a recording of the colour differences

At the age of 13 months the rats were used for acute experiments First resting blood pressure was measured directly in the intact anesthetized animals (Nembutal 40 mg/kg i p) by means of a cannula inserted into the tail artery Utilizing the technique of artificial paired perfusion (Folkow *et al* 1970 b) the isolated hindquarters of 12 SHR_{is} and 8 NCR_{is} were then perfused at constant flow rate in parallel with those of the normotensive control rats (NCR) that had been injected with 0 9 % NaCl at birth or with those of other matched NCR The earlier in detail described procedure will here be only briefly summarized The hindfeet were tied off in order to get a more pure muscle preparation the vascular bed of the tail being already obstructed by the arterial cannula in which the perfusion pressure was continuously measured After isolation of the hindquarters from the upper part by standardized mass ligatures the abdominal aorta was cannulated 1 cm proximally to the bifurcation The inferior caval vein was widely opened to permit free outflow and the perfusion was started at a constant rate of approximately 10 ml/min × 100 g tissue throughout the experiment Oxygenated Tyrode solution was used as perfusate containing 4 % Ficoll (a polymer of sucrose and epichlorohydrin kindly supplied by AB Pharmacia Sweden to furnish a suitable colloidosmotic pressure When maximal vasodilatation had been obtained by slug injections of papaverine constant infusion of noradrenaline (NA) was started at subthreshold concentrations increasing in a stepwise fashion until the maximal pressor response was reached as secured by slug injections of vasopressin (10 IU) and $BaCl_2$ (150 mg at the end Flow resistance at maximal dilatation and the resistance responses to NA vasopressin and $BaCl_2$ were calculated for each pair of hindquarter preparations and the resulting resistance curves were plotted with the log NA dose per ml perfusate on the abscissa and the pressor (resistance) response on the ordinate For details concerning the characteristics of these curves and their interpretation see Folkow *et al* 1970 b

Results

When compared with ordinary SHR the treatment had reduced arterial blood pressure in SHR_{is} by some 10 % as measured indirectly and intermittently for the

[1] Generous gift by the Wellcome Reagents Ltd Beckenham Kent England

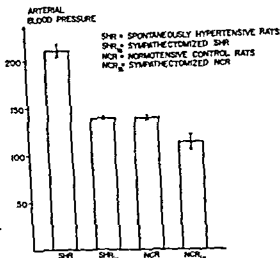


Fig 1 "Resting" blood pressure of NCR SHR NCR$_{is}$ and SHR$_{is}$, as measured directly *via* a cannula in the tail artery during standardized Nembutal anesthesia at the time for the perfusion experiments

first 8 months by the tail calorimetric method However, the pressure of SHR$_{is}$ was during this period as an average 20—25 % higher than that of NCR$_{is}$ but about the same as in ordinary NCR Further, when blood pressure was measured directly in the different groups of animals *via* the cannulated tail artery during standardized Nembutal anesthesia just before the perfusion experiment was started the same differences, or similarities, in pressure were observed Thus, the mean arterial pressure values in SHR$_{is}$ and NCR$_{is}$ were 139±1 and 113±2, respectively For untreated SHR and NCR of the same age, mean blood pressure in the caudal artery, measured under identical circumstances as in SHR$_{is}$ and NCR$_{is}$ was 201±8 and 139±2, respectively (see Fig 1), *i e* SHR$_{is}$ and ordinary NCR had largely the same pressures, also when these were measured intraarterially

The marked interference with normal adrenergic fibre control in SHR$_{is}$-NCR$_{is}$ were, apart from the pressure reductions obvious also by the presence of enophtalmus and by the fact that the treated animals exhibited no sign of reflex vasodilatation in the tail upon indirect warming, they were, on the whole very sensitive to *e g* heating indicating an interference with the thermoregulatory control of cutaneous blood flow Administration of a ganglionic blocker (Ecolid® Ciba) produced a prompt and marked fall in blood pressure in ordinary SHR and NCR while the same administration in SHR$_{is}$ and NCR$_{is}$ produced a much delayed and very slow pressure reduction which was only moderate in extent The characteristics of this pressure fall in SHR$_{is}$-NCR$_{is}$ indicated that it was mainly a consequence of a blockade of the adrenal medullary secretion It is known that the adrenal medullary cells are hardly affected by the nerve growth factor antiserum (Zaimis 1967) and it appears that this part of the sympatho-adrenal system may represent practically the only adrenergic excitatory influ

SHR$_{is}$ and NCR$_{is}$

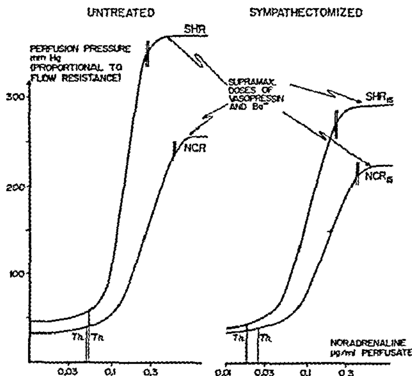


Fig 2 Average "resistance curves for ordinary NCR and SHR (left part) and for NCR and SHR, right part with log NA dosage along the abscissa and resistance i e the pressor response at constant flow perfusion along the ordinate The shaded areas represent the differences between the hypertensive and normotensive resistance curves as to their various "key points see Folkow *et al* 1970 b Fig 2) These differences between the two groups of curves are in principle the same whether the sympathetic cardiovascular supply is intact (left part) or largely destroyed right part) though less pronounced in the sympathectomized animals and in proportion to their reduced difference in blood pressure

From the results of the 20 paired perfusion experiments mean resistance curves were calculated for the different groups of rats (Fig 2) Since one NCR was always perfused in parallel with one SHR or one NCR the differences between these treated groups of animals were calculated from their difference to NCR The mean resistance curve for the entire group of NCR used in these paired perfusions is presented in the left part of Fig 2 which also illustrates the *percentage* difference between NCR and ordinary SHR as obtained by pooling data from a previous study (Folkow *et al* 1970 b and from more recent direct comparisons between NCR and SHR The right part of Fig 2 illustrates the relationships between the resistance curves of SHR, and NCR which as mentioned differed 20—25 % with respect to resting blood pressures Compared with NCR, SHR showed 18 % increase of resistance at maximal dilatation 42 % increase in steepness of the slope of the resistance curve and 26 % increase in maximal pressor response these differences

TABLE I The table illustrates mean values ($\pm$S E) of NCR, SHR_{is} and NCR_{is} and the significance levels of the differences between the three groups (The NCR resistance value at maximal vasodilatation given here is somewhat higher than that given by Folkow *et al* 1970 b, which is explained by the fact that the richly vascularized tail and feet were excluded in the present preparation and the viscosity of the perfusate was somewhat higher)

	Arterial blood pressure mm Hg	PRU_{100} at max.dil	Threshold µg NA/ml	Corrected slopes of curves as tangent of the angle	Maximal pressor response
NCR n = 20	139±2	3.2±0 8	0 051±0 08	3 0±0 2	254±7
SHR_{is} n = 12	139±1	3 4±0 1	0 018±0 006	3 7±0 3	279±9
NCR_{is} n = 8	113±8	2 9±0 2	0 025±0 005	2 6±0 2	221±10
Significance					
NCR/SHR_{is}	—	—	p < 0 001	p < 0 005	p < 0 001
NCR/NCR_{is}	p < 0 05	—	p < 0 05	—	p < 0 01
SHR_{is}/NCR_{is}	p < 0 05	p < 0 05	—	p < 0 005	p < 0 001

being all statistically significant or highly significant There was on the other hand, no statistically significant difference between SHR_{is} and NCR_{is} with respect to NA threshold , defined as the NA dosage that produced a 25 % increase in resistance from that at maximal dilation (see also Table I which presents the average values for SHR_{is}, NCR_{is} and the ordinary NCR used in the present experiments) Thus despite the extensive destruction of cardiovascular adrenergic nervous control in SHR_{is} and NCR_{is} the characteristic differences between the resistance curves of ordinary SHR and NCR (see Folkow *et al* 1970 b, illustrated also in the left hand part of Fig 2) were also seen in these treated animals though to a reduced extent and largely in proportion to the respective differences in ' resting blood pressure

In Fig 3 the average resistance curve of SHR_{is} is directly compared to that of the untreated NCR these two groups of animals exhibiting the same resting blood pressure level in the present series of experiments This Figure illustrates that these two groups of animals display the following differences with respect to the characteristics of their resistance curves In SHR both the steepness of the resistance curve and the maximal pressor response are increased compared with NCR and even though these differences are not a pronounced as those between SHR_{is} and NCR_{is} they are still statistically significant or highly significant (Table I There is also a tendency of the resistance at maximal dilatation to be higher in SHR_{is} than in NCR but this difference is not statistically significant The NA threshold is on the other hand significantly increased in SHR_{is} compared to NCR thus revealin a moderate degree of supersensitivity of the SHR_{is} resistance vessels

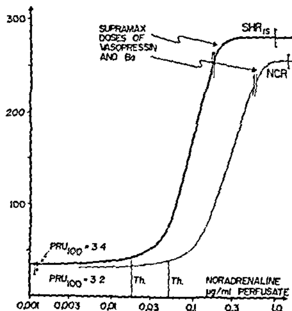


Fig 3 Average resistance curves for SHR$_{IS}$ and ordinary NCR The shift to the left of the SHR$_{IS}$ curve reflects the denervation hypersensitivity Even though these two groups of rats had equal resting blood pressures the SHR$_{IS}$ curve displays an increased resistance at maximal dilatation though not statistically significant a significantly increased slope of the curve and a significantly increased maximal pressor response as compared to NCR (see text and Table I)

Discussion

Previous hemodynamic comparisons between SHR and NCR, and studies in man strongly suggest the presence of a structurally enhanced wall/lumen ratio of the resistance vessels in at least primary hypertension evidently a result mainly of an adaptive media thickening that encroaches upon the vascular lumen even at complete relaxation of the contractile elements (for details see Folkow, Grimby and Thulesius 1958 Sivertsson 1970 Folkow *et al* 1970 a b Folkow 1971 a) Both in SHR and in essential hypertension in man this type of structural adaption seems to bee so closely tailored to the pressure level as to being alone able to explain largely the entire rise in systemic resistance without necessitating any enhanced vascular smooth muscle activity at least in the resting equilibrium

Even though such a structural vascular adaptation developing within few weeks as a local response to a change in average transmural pressure (Folkow and Sivertsson 1968, Folkow *et al* 1971 a) seems to be a prerequisite for creating a truly hypertensive system, its initiation evidently calls for some type of functionally initiated pressure load Studies by particularly Okamoto and his group on SHR (Okamoto 1969) strongly suggest that the sympathetic nervous system plays an important and genetically linked, role in this type of primary hypertension in rats However a raised sympathetic influence may well initiate structural vascular changes also if its impact is intermittent provided that the integrated pressure load is substantially raised for a sufficient period of time Such a type of neurogenic influence appears

as the more likely alternative because there is in fact, no clear evidence of any significantly raised smooth muscle activity of the systemic resistance vessels in the "resting" equilibrium of primary hypertension either in SHR or in man. Thus basal vascular tone i.e. the ratio between the resistance at rest and that at maximal dilatation (see e.g. Sivertsson 1970), proves to be largely the same in hypertensives as in normotensives during rest.

Several earlier studies illustrate that also intermittent sympatho adrenal exacerbations can, indeed gradually lead to a more persistent increase of blood pressure. For example weak topical stimulations of the hypothalamic defence area, normally conveying the autonomic cardiovascular adjustments induced by alerting environmental stimuli can cause a gradual rise of "resting" blood pressure in rats when repeated frequently for some months (Folkow and Rubinstein 1966). Further mice, living in an environment where stressful encounters are frequent can gradually develop hypertension and subsequently the full spectrum of cardiovascular lesions associated with more pronounced hypertension (Henry, Meehan and Stephens 1967). Psychosocial factors may be of considerable importance also in essential hypertension in man (e.g. Charvat, Dell and Folkow 1964, Henry and Cassel 1969) and the early labile phase of this disorder of regulation often displays a cardiovascular pattern apparently identical with that of a mild defence reaction, the pressure rise being associated with an increased cardiac output and an enhanced muscle blood supply (e.g. Brod 1963, Sannerstedt 1966).

Towards such a general background the present experiments were designed to explore what will happen if the impact of the sympathetic nervous system is drastically interfered with already in early stages of life by means of immunosympathectomy. Both treated and untreated SHR and NCR were compared first concerning the development of the "resting" level of arterial blood pressure and second concerning the structural design of the resistance vessels, here judged by quantitative hemodynamic comparisons. For evaluations of the hemodynamic impact of an altered vascular design such an approach has several great advantages over morphological measurements. First the distending pressure can be exactly monitored which is crucial when distensible tubes are to be compared. Second the resistance vessels can be studied both when their contractile elements are completely relaxed, when they are maximally contracted against a known load and when they are kept at comparable intermediate levels of activity. Third any alteration of luminal dimensions, however brought about becomes greatly amplified when explored in terms of resistance changes. The reason is of course that resistance varies inversely to the fourth power of the internal radius which means that only e.g. a 5 per cent radius change—difficult if not impossible to measure directly—causes more than 20 per cent change in resistance which is easy to measure.

To interfere with sympathetic cardiovascular control, immunosympathectomy was used which in both SHR and NCR prevented the blood pressure from reaching the levels usually achieved in these animals. In SHR, resting blood pressure was largely the same as in ordinary NCR, in agreement with recent findings in the New

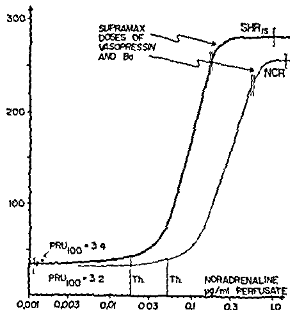


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To interfere with sympathetic cardiovascular control, immunosympathectomy was used which in both SHR and NCR prevented the blood pressure from reaching the levels usually achieved in these animals In SHR_i, resting blood pressure was largely the same as in ordinary NCR in agreement with recent findings in

Zealand strain of genetically hypertensive rats, exposed to immunosympathectomy (Clark 1971) Clark interpreted his results as indicating that this latter type of hypertension in rats which appears to be less severe than that in SHR, was of essentially neurogenic origin

Though neurogenic factors are no doubt most important also in SHR the present results suggest that additional factors, which may be of considerable relevance also for the genetic linkage, should also be considered It seems, for example, remarkable that SHR_{is}, though largely deprived of normal adrenergic nervous control, were still able to maintain the same resting blood pressure as untreated NCR where neurogenic mechanisms no doubt contribute considerably to the maintenance of pressure Further, SHR_{is} exhibited a clearly higher resting blood pressure than NCR_{is}, though this difference was smaller (about 25 %) than that between untreated SHR and NCR (about 40—45 %) Thus already such circumstances seem to suggest that additional factors are involved in maintaining a higher pressure in SHR, besides that inherent in the adrenergic control mechanisms Possible differences between SHR_{is} and NCR_{is} as to sympatho-adrenal mechanisms should, however, first be considered

It is possible first, that SHR_{is} exhibit a higher sensitivity to catecholamines, second that the sympathectomy was less efficient in SHR_{is} and, third that their remaining adrenergic mechanisms display an enhanced activity However, NA 'threshold' did not differ significantly between SHR_{is} and NCR_{is}, in agreement with ' er comparison between ordinary SHR and NCR (Folkow *et al* 1970 b Hall ' Lundgren and Weiss 1971) which makes it less likely that the difference n pressure between SHR_{is} and NCR_{is} should be a mere consequence of a difference in NA sensitivity As to a possible difference in the extent of sympathectomy, it is known that immunosympathectomy is in general never quite complete and the hormonal link in terms of the adrenal medullary cells appears to remain largely unaffected (Zaimis 1967 Clark 1971) Should SHR_{is} and NCR_{is} differ in this respect *e g* due to a higher resistance of SHR to the antiserum with better preserved nervous connections as a result some difference as to NA sensitivity would be expected and also a more sudden onset in the pressure fall in SHR_{is} when ganglionic blockers were given (see Results) However these observations rather suggested that largely the entire adrenergic control in SHR_{is} seemed to be of hormonal origin

Concerning the possibility of an enhanced activity in remaining adrenergic control mechanisms it appears that the adrenal medullary secretion is of only minor relevance for normal reflex homeostasis in the resting organism It is, however, often pronounced in connection with activation of the hypothalamic defence area, which normally conveys the cardiovascular adjustments in response to a variety of alerting and stressful stimuli In case SHR for genetic reasons display a somewhat enhanced 'reactivity' of such central autonomic structures (*cf* Okamoto 1969), the preserved hormonal link of the sympathetic system might be enough to convey a relatively more intense excitatory drive on the cardiovascular system in SHR_{is} than in NCR_{is} As in ordinary SHR and NCR such a type of excitatory influence may, even if

intermittent in nature imply a somewhat higher average pressure load on the SHR_s vessels

Such a factor might act as a functional trigger mechanism and hence explain also the clear difference between SHR_{is} and NCR_s with respect to structural design of their resistance vessels as suggested by the differences between their respective resistance curves These characteristic differences are, in fact the same as those between ordinary SHR and NCR but reduced in extent in close proportion to the less prononunced difference in resting blood pressure Thus the ratio of resting blood pressure between SHR_s and NCR_s was 1 23, while the ratios for PRU_{100} at maximal vasodilatation was 1 18 for the slope of the steep part of the resistance curve 1 42 and for the maximal pressor response 1 26 The corresponding ratios in ordinary SHR NCR were 1 45 1 35 1 55 and 1 45 respectively *i e* a very close correlation to the differences in blood pressure between the treated and untreated animals

Whichever differences there may exist between SHR_s and NCR_s with respect to their grossly reduced neuro-hormonal control of the circulation it thus seems clear that they differ in vascular design in a way which may largely explain their difference in resting blood pressure as is the case in ordinary SHR and NCR (for details see Folkow *et al* 1970 b) The question is whether the ability to respond with structural adaptation is basically the same in SHR and NCR though the changes become more pronounced in SHR simply because neuro hormonal influences are here for genetical reasons more powerful

It should be stressed however that SHR and NCR might genetically differ also in the sense that the cardiovascular tissues of SHR are slightly more prone to respond with hypertrophy when exposed to a given load Even a small quantitative difference in this respect may in the long run exercise a considerable influence particularly since structural and functional changes would tend to mutually reinforce each other as to vascular control (see *e g* Fig 2 Folkow *et al* 1970 b or Folkow 1971)

A genetically linked factor of this nature has earlier been considered with respect to essential hypertension in man (Folkow Grimby and Thulesius 1958 Sivertsson 1970) It was one of the primary aims of the present study to try to shed some light on this possibility as to the primary hypertension in SHR It then seems to be of particular interest that SHR_s despite the indisputable and marked interference with their sympathetic cardiovascular control were able to maintain the same resting blood pressure as untreated NCR with their full neurogenic command of the circulation This was so both in the awake animals during rest and after standardized anesthesia Moreover at similar resting blood pressures SHR displayed some clear signs of altered structural design of their resistance vessels as compared with NCR and in the hypertensive direction (Fig 3 and Table I) Thus SHR showed a slight though significant increase of the slope of the resistance curve and a highly significant though modest increase of the maximal pressor response when compared to the normally innervated vascular bed of NCR. Both these differences are indicative of a somewhat larger bulk of media tissue in SHR, than in NCR (for d

see Folkow *et al* 1970 b) though this wall change is not large enough to encroach upon the lumen to a statistically significant extent, comparing SHR_{ii} with NCR, even if a tendency in this direction can be traced

In other words, when compared with ordinary NCR, SHR_{ii} still appears to display some of the "stigmata" characterizing a hypertensive vascular bed although the blood pressure levels would suggest largely equal pressure "loads' on the vascular walls Again, such a structural difference might explain why SHR_{ii} can maintain the same resting blood pressure as NCR (see *e g* Fig 2, Folkow *et al* 1970 b), despite a reduced sympatho adrenal contribution to resting vascular tone in SHR, Therefore, the present results indicate that the often massive hypertension in SHR might be genetically linked to both a functional and a structural element where each may be considered as a *per se* ' normal' variant It should be stressed that even fairly small deviations from the mean along a Gaussian curve of ' normality", as to such a structural factor, may in the long run have considerable hemodynamic consequences since it appears likely that functional ' trigger" mechanisms and structural adaptations of the resistance vessels tend to mutually reinforce each other

... rg and ... generous ... len for ... Sweden
has generously covered part of the expenses for technical assistance

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Hedgehog Monoamine Oxidase

By

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Abstract

Blaschko, H *Hedgehog monoamine oxidase* Acta physiol scand 1972 *84* 524—527

Homogenates of the liver the kidneys and the brain of the hedgehog *Erinaceus europaeus*, have been tested for monoamine oxidase activity The enzyme was present in all 3 tissues, and the enzyme was found to act on all the substrates known to be oxidised by other mammalian sources of the enzyme When the rates of oxidation of tyramine and β phenylethylamine were compared, it was found that the relative rate of the latter amine in liver was about 3 times that found for kidneys In the brain the position seems to be intermediate

e comparative study of an enzyme in different animal species, as well as in different organs of the same species has often yielded interesting results Instances are known where differences in substrate specificity are parallelled by differences in the chemical composition of the enzyme protein

For the enzyme monoamine oxidase (MAO) both types species and organ differences, have been known for some time A recent study reveals examples that have been observed in two species of Cyclostomes, *Petromyzon marinus* and *Myxine glutinosa* (Blaschko, Boadle and Strich 1969) The liver of *Petromyzon* yields an enzyme capable of oxidising a great variety of substrates, on the other hand *Myxine* has an enzyme with a much more restricted substrate specificity Also the pattern of substrate specificity of the *Petromyzon* kidney enzyme is much narrower than that of *Petromyzon* liver

Another well studied example of differences in organs of one and the same species occurs in the mouse (Hagen and Weiner 1959 Hope and Smith 1960)

These observations have gained in interest in recent years when observations were reported which suggest the existence of isoenzymes of MAO with differing substrate specificities (see *e g* Johnston 1968 Kim and D Iorio 1968, Youdim, Collins and Sandler 1969) On the basis of such an interpretation organ differences in substrate specificity might be considered as due to the presence of the different isoenzymes of MAO in differing proportions

During a stay at the Institute of Physiology, Bergen University, in September 1970 it became possible to test some of the tissues of the hedgehog *Erinaceus europaeus* for MAO activity Use was made of this opportunity since no information is available on the properties of MAO in the Order Insectivora

Methods

Homogenates were usually prepared using fresh tissue but occasionally also after the organ had been kept deep-frozen overnight. The tissues liver kidney or brain were weighed and homogenized in 0 067 M Sorensen phosphate buffer of pH 7 4 the final volume was adjusted so that to 1 g of tissue 2 ml of buffer were added The homogenates were briefly centrifuged at very low speed in order to remove connective tissue and coarse debris they were then dialysed with stirring usually overnight, against large volumes of the same buffer The latter was changed at least twice

In the manometric experiments suitable amounts of tissue were further diluted with buffer if necessary The amines were tested as hydrochlorides with an initial concentration of 10^{-2} M The temperature of the manometer bath was 37 5°, and the gas phase was O_2

The enzymic activities have been calculated in terms of qO_2 *i e* as μlO_2 consumed per mg of tissue per hour

Results

Typical MAO activity was found in all 3 tissues tested Liver kidney and brain acted on tyramine β phenylethylamine benzylamine and isoamylamine In addition the liver homogenate acted on β phenylethanolamine, 1 10-diaminodecane and tryptamine these amines were not tested with the two other preparations The figures for qO given in Table I show that the enzymic activity of the 3 tissues examined was high

The fact that the long-chain diamine 1 10-diaminodecane, was oxidised, is in keeping with what is known of MAO from other mammalian sources In an experiment with hedgehog liver hexamethylenediamine was not oxidised no oxidation of putrescine was seen with the brain homogenate The rate of oxidation of isoamylamine particularly by the liver extracts was remarkably high

In Table I the results obtained with the various amines are expressed in terms of the ratio rate of oxidation of tyramine to rate of oxidation of the other amine tested Tyramine was used in every experiment in order to make possible a comparison of the different experiments with each other

The agreement between the results obtained in the different experiments is satisfactory The most interesting result of the present experiments is in the relative rate of oxidation of β phenylethylamine in the three tissues studied Inspection of Table I suggests the possibility that the relative rates of oxidation of isoamylamine in kidney and liver were also different but more experiments would have to be carried out in order to establish this point securely

On the other hand there can be little doubt that the relative rates of oxidation tyramine to β phenylethylamine are different for the tissues tested The difference is most marked when the results for liver and kidney are compared the ratio for the

TABLE I Relative rates of oxidation of various amines by homogenates of hedgehog tissues

The relative rates of oxidation are expressed in terms of the ratio rate of oxidation of tyramine rate of oxidation of the other amine tested The readings obtained during the first 15 min periods and the first 30 min periods respectively are used

Ty tyramine IAA isoamylamine Bz benzylamine β PhEt β-phenylethylamine, β PhEtoh β phenylethanolamine, C_{10} 1 10 diaminodecane, Try tryptamine

Enzymic activity with tyramine as substrate is expressed in terms of qO_2 i e in μl O_2 consumed per mg of fresh tissue per hour (Not corrected for volume changes during dialysis)

Exp	Organ	qO_2(Ty)		Ty/IAA		Ty/Bz		Ty/β PhEt		Ty/β PhEtoh		Ty/C_{10}		Ty/Try	
No		15	30	15	30	15	30	15	30	15	30	15	30	15	30
1	Liver	1 09	1 04	—	—	3 2	4 2	—	—	—	—	—	—	—	—
2		1 05	0 99	—	—	—	—	2 2	3 4	—	—	—	—	—	—
3		1 00	0 98	—	—	—	—	1 95	2 9	5 9	4 9	21 0	13 2	—	—
4		0 96	0 84	—	—	2 9	3 6	—	—	—	—	—	—	—	—
6		1 01	0 94	1 1	1 4	—	—	—	—	—	—	—	—	4 5	7 3
6	•	1 03	0 98	1 4	1 7	—	—	—	—	—	—	—	—	—	—
7	Kidney	0 23	0 25	1 9	2 2	3 5	4 0	7 6	9 2	—	—	—	—	—	—
8		0 21	0 24	2 2	2 75	—	—	5 8	9 6	—	—	—	—	—	—
9	Brain	0 41	0 48	1 65	2 3	3 1	5 1	3 9	7 4	—	—	—	—	—	—

kidney is about 3 times that for the liver In other words the relative rate of oxidation of β phenylethylamine in the liver is very high

It seems likely that the true initial rate of oxidation of β phenylethylamine may even have been a little higher The increasing ratios from the 15 min to the 30 min period are due to a fall off of the rate of oxidation of β phenylethylamine this fall off began already before the end of the first 15 min period of observation

Discussion

The fact that the 3 tissues of the hedgehog contain MAO is scarcely worthy of comment The activity of the tissues is high and the overall properties of the enzyme conform to the general mammalian pattern The rate of oxidation of isoamylamine seems unusually high

What is interesting is a result that has already been mentioned the relative rate of oxidation of β phenylethylamine The observations already referred to in the introductory part, on isoenzymes of MAO suggest one possible explanation of the results obtained different isoenzymes of MAO might exist in the hedgehog with widely different ratios of activity against tyramine and β phenylethylamine These isoenzymes may be present in differing proportions in liver and kidney and possibly also in brain Alternately, the possibility must be considered that in these tissues there exist varieties of the enzyme which express themselves in a different activity towards different substrates These are possible interpretations which will only be fully elucidated when the MAO from different sources has been fully purified

All these experiments were carried out in the course of a few weeks, in September, on a limited number of specimens MAO is an enzyme known to be susceptible to the action of various hormones The hedgehog is a species in which seasonal changes are especially marked It would be of interest if the differences uncovered in the present study present any seasonal differences

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It [illegible]
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my c [illegible]
Brodtkorb and to Mrs Straus for skilful technical assistance

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Acta physiol. scand 1972. 84. 528—537

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Influence of Extracellular Calcium on Isometric Force and Velocity of Shortening in Depolarized Venous Smooth Muscle

By

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Abstract

HELLSTRAND P B JOHANSSON and A RINGBERG *Influence of extracellular calcium on isometric force and velocity of shortening in depolarized venous smooth muscle* Acta physiol scand 1972 *84* 528—537

The Ca^{++} dependence of the contracture response obtained in depolarized smooth muscle from rat portal vein was studied Double reciprocal plots of isometric force versus $[Ca^{++}]^n$ were examined by linear regression analysis for n = 1, 2 and 3 The data were consistent with a linear relationship for n = 2 This may be taken to indicate that isometric force is regulated by the binding of two calcium ions to an unknown receptor compound in the tissue In other experiments the velocity of shortening was studied by the method of quick release after the muscles had developed isometric contractures in 2 5 or 0 4 mM $[Ca^{++}]_o$ respectively The mean isometric forces elicited by these 2 levels of $[Ca^{++}]_o$ differed by a factor of 1 7 but the shortening velocities at comparable relative loads were essentially the same These results may be considered in the light of recent studies of striated muscle which have been concerned with the problem whether Ca^{++} determines exclusively the number of activated cross bridges in the sliding filament system or whether it also influences the rate of movement of the individual cross bridge

The spontaneous electrical and mechanical activity of the isolated rat portal vein is rapidly abolished in nominally Ca free Krebs solution (Axelsson *et al* 1967) Moreover, depolarization of the smooth muscle by high K fails to induce contraction after only short periods (10 to 20 min) in the Ca free medium indicating that intracellular calcium stores cannot be mobilized to activate the contractile system under these conditions If Ca is now added to the depolarizing solution a sustained contracture develops and the magnitude of this response is intimately dependent on $[Ca^{++}]_o$ (Biamino and Johansson 1970) It seemed that a detailed analysis of the contracture response as a function of extracellular [Ca] would provide useful quantitative information about the role of Ca in smooth muscle contraction The present study is concerned with this problem

Two types of experiments have been performed *1 Recording of isometric force* in Ca induced contractures, *2* Recording of isotonic shortening against different

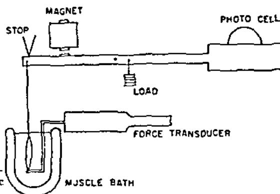


Fig 1 Schematic illustration of apparatus used for studying isotonic shortening after quick release

loads following quick release The results confirm the intimate Ca -dependence of the isometric force and indicate a linear relationship between the reciprocal of the active force and the reciprocal of [Ca]$^{+}$ By contrast the velocity of shortening at any given relative load appears to be independent of [Ca^{++}]

Methods

Segments of the rat portal vein 4 to 8 mm long were dissected cut open longitudinally and mounted in the organ bath In the isometric experiments the muscles were stretched to a passive force of about 400 dyn This gives approximately the muscle length which is optimal for active tension development In the isotonic experiments it was important to minimize the possible influence of parallel elastic elements on the rates of shortening and passive tension was therefore kept as low as 50 to 75 dyn

... min in a modified Tris- ... 15 KCl 6 0 MgCl$_2$ 1 2, ... ma Base Sigma Chemi ... 7 4 at 37° C and in the ... ommodation period the muscle developed a regular spontaneous activity with 2 to 4 phasic contractions per min a stable resting tension prevailing in the intervals between the contractions(cf Axelsson *et al* 1967)

In the isometric experiments contractile force was recorded by means of a Grass FTO3 force transducer and a direct writing oscillograph (Grass Polygraph) The experimental protocol was as follows After the accommodation period the muscles were exposed to Ca^{++} free normal solution by which spontaneous activity was abolished in 5 to 10 min The ability of depolarization to evoke contraction was now tested by shifting to a solution containing 106 mM K^{+} This had the same composition as the Ca free normal solution except that 100 mM NaCl had been replaced by KCl The exposure to the Ca^{+} free depolarizing medium lasted for 5 min after which Ca^{+} free normal solution was readministered Isometric force was recorded at high sensitivity and a weak to moderate contraction was observed in response to the first depolarization following the withdrawal of Ca^{+} However after two or three periods of exposure to the depolarizing medium tension did not deviate significantly from resting tension indicating satisfactory removal of Ca The true experiment could then be started according to the following schedule After 10 min in Ca^{++} free normal solution the muscle ... $CaCl_2$ was added to ...

min in this medium. Contracture tensions in response to graded Ca concentrations from threshold (0 05 to 0 1 mM) up to 20 mM were studied according to this rigid time schedule in a total of 6 preparations Temperature was kept at 37° C throughout the experiments

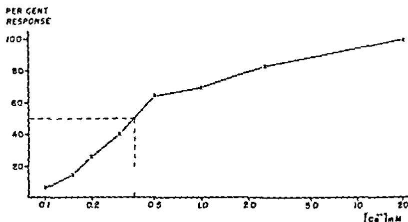


Fig 2 Contracture tension of depolarized portal vein as a function of extracellular [Ca^{++}]. Data from 1 exp Determination of the approximate "[Ca^{++}]$_{50}$" indicated by the interrupted line

The apparatus used in the isotonic experiments is shown schematically in Fig 1 A force transducer Grass FTO3 was connected to the lower end of the muscle by means of a rigid steel rod The upper end of the muscle was connected to an isotonic lever through a straightened steel wire A photoelectric device was used to record the movements of the lever The latter was also supplied with a small dashpot device for damping of oscillations (not shown in Fig 1) Variable loads could be applied on the lever arm opposite to the muscle attachment the lever arm of the muscle being 6 times that of the load The isotonic lever could be locked by a stop screw and an electromagnet so that the muscle contracted isometrically

The equivalent mass of the isotonic lever was found to be about 2 5 g The system would therefore be unsuitable for studying shortening velocity in fast muscles but appears acceptable for the present experiments on smooth muscle As judged by the constancy of the force following quick release (see below the inertia of the isotonic lever was of little importance during the phase of the length change that was used for measuring the shortening velocity of the contractile element (Fig 4

The preload and the position of the stop screw were adjusted at the beginning of the experiment so as to give a passive tension of 50 to 75 dyn on the resting muscle

Removal of Ca^{++} and depolarization of the smooth muscle were done as described above [illegible] ked by addition of 0 4 or 2 5 mM Ca^{++}, respectively [illegible] $^{++}$ concentrations could be expected to give isometric [illegible] wo approximately [illegible] ectromagnet the muscle could be abruptly released and allowed to shorten against loads which corresponded to variable fractions of the isometric force

The signals from the force transducer were recorded on a Grass polygraph The changes in muscle length as detected by the photocell and the first time derivative of these length changes as obtained by an electronic differentiator (time constant 47 ms) were recorded on moving film by means of a double beam oscilloscope Tectronix 502A) and a Grass kymograph camera. Paper and film speed respectively were kept equal in these recordings

Results

1 Contracture tension as a function of [Ca^{++}]$_o$

Fig 2 summarizes the results from one of the isometric experiments Particular emphasis has been placed on careful determination of the contractile responses to the low Ca^{++} concentrations which influence very much the configuration of the

[Ca^{++}] response curve The force is given as a percentage of the contractile force obtained at a Ca -concentration of 20 mM, which appears to give a maximal response Control experiments showed that responses to 50 mM Ca did not differ significantly from those obtained with 20 mM, and 100 mM often gave somewhat lower contracture tensions

The curves relating contractile response to [Ca^{++}], were remarkably steep in all the experiments, and it was considered of interest to subject them to further analysis It seems appropriate, therefore, to introduce at this stage the following assumptions

(i) The curves reflect the binding of Ca^{++} to an unknown receptor substance', R



$$n\ Ca^{++} + R \leftrightarrows RCa_n \tag{1}$$

The integer n thus indicates the number of Ca^{++} ions bound to each receptor

(ii) The tension response E, is directly proportional to the amount of Ca receptor compound

$$E = \text{const}\ [RCa_n] \tag{2}$$

Let K_n be the dissociation constant for reaction (1)

$$\frac{[Ca^{++}]^n\ [R]}{[RCa_n]} = K_n \tag{3}$$

R_T is introduced to denote the total concentration of receptors

$$[R] = R_T - [RCa_n] \tag{4}$$

By inserting eq (4) into eq (3) and rearranging we get

$$\frac{R_T}{[RCa_n]} = \frac{K_n}{[Ca^{++}]^n} + 1 \tag{5}$$

The maximal response E_{max} is obtained for $[RCa_n] \sim R_T$ Eq (2) and (5) then give

$$\frac{E_{max}}{E} = \frac{K_n}{[Ca^{++}]^n} + 1 \tag{6}$$

The constant K_n is seen to represent the value of $[Ca^{++}]^n$ at which half the maximum isometric tension is obtained

According to eq (6) a plot of E_{max}/E versus $[Ca^{++}]^{-n}$ should give a straight line provided n is chosen correctly Fig 3 shows the data from the experiment of Fig 2 plotted in this way The constant n has been given the values 1 2 3 respectively Responses below 5 per cent of maximum are uncertain and have been excluded as possibly giving large errors when placed in the denominator Regression lines for the three sets of points were determined by the method of least squares If the above assumptions are valid the regression line which conforms to the following criteria should indicate the appropriate value of n

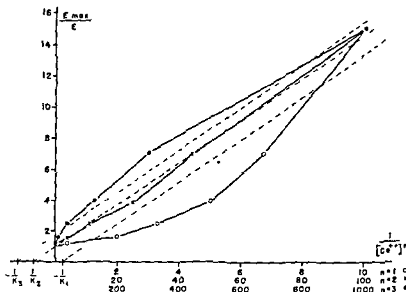


Fig 3 Double reciprocal plots of response versus $[Ca^{++}]^n$ for n = 1, 2 and 3 respectively Same experiment as in Fig 2 The interrupted lines are the regression lines calculated by the method of least squares

1 its intersection with the ordinate should be unity

2 its intersection with the abscissa (at $-1/K_n$) should be related to the $[Ca^{++}]$ for 50 per cent response by $\sqrt[n]{K_n} = [Ca^{++}]_{50}$,

3 its correlation coefficient should approach +1

The results of the six experiments in this group, analysed in this way, are presented in Table I With the three integral values of n which have been considered, the table gives the following data for the respective regression lines intersection with the ordinate (b), slope (m), correlation coefficient (r) and $\sqrt[n]{K_n}$, which may be compared with the $[Ca^{++}]_{50}$ determined directly from the Ca^{++} response curve (cf Fig 2)

It is evident from Table I that the regression lines calculated for n=2 fulfil the above criteria better than the lines calculated for n=1 or n=3

2 *Relationship between force and velocity of shortening*

Fig 4 shows a series of recordings of force and shortening obtained when an isometrically contracted muscle was suddenly released with different loads applied to the isotonic lever The isometric contracture tension (P_0) of this muscle was about 550 dyn in the three records of Fig 4 Some initial oscillations due to inertia followed the quick release but the force then stabilized at levels corresponding to 70,

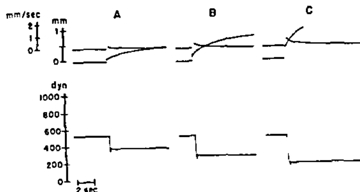


Fig 4 [illegible]

[illegible] first time derivative of the muscle shortening

53 and 31 per cent of P_o, respectively The changes in muscle length can be separated into three fairly distinct phases *1* a rapid immediate phase mainly due to recoil of the series elastic elements *2* damped inertial oscillations *3* a relatively slow phase representing shortening of the contractile elements This interpretation of the quick release curves is identical to that given by other workers who have applied the method to skeletal or heart muscle (*e g* Jewell and Wilkie 1958, Edman and Nilsson 1968) It is evident from Fig 4 that the rate of shortening of the contractile elements decreases with a larger relative load but the exact quantitative comparison is complicated by the fact that shortening velocity gradually falls off over the entire

TABLE I Characteristics of regression lines for the double reciprocal plot of response (E) versus $[Ca^{++}]^n$ according to the equation $\frac{E_{max}}{E} = m \frac{1}{[Ca^{++}]^n} + b$

The table [illegible]

	b			m			r			$\sqrt[n]{K_n}$			$[Ca^{++}]_{50}$
n	1	2	3	1	2	3	1	2	3	1	2	3	
Muscle no													
1	−0 17	1 15	1 77	1 20	0 13	0 013	0 9404	0 9963	0 9878	−7 07	0 34	0 19	0 35
2	−0 16	1 08	1 68	1 17	0 13	0 013	0 9287	0 9953	0 9945	−7 32	0 35	0 20	0 31
3	−0 40	1 01	1 68	1 30	0 14	0 014	0 9426	0 9984	0 9915	−3 25	0 37	0 20	0 37
4	0 07	1 00	1 44	0 88	0 10	0 010	0 9197	0 9973	0 9977	12 57	0 35	0 19	0 32
5	−0 31	0 82	1 31	0 84	0 09	0 009	0 9170	0 9885	0 9747	−2 74	0 33	0 19	0 26
6	−0 44	0 94	1 52	1 03	0 11	0 011	0 9156	0 9886	0 9985	−2 34	0 34	0 10	0 33

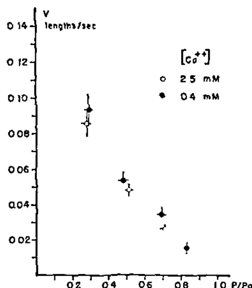


Fig 5 Diagram showing velocity of shortening in muscle lengths/sec as a function of relative load for contractures elicited by 2 5 and 0 4 mM Ca , respectively The data have been grouped into 4 equal classes on the basis of P/P between 0 2 and 1 0 The diagram shows mean ± S E for 6 muscles examined at both levels of [Ca++]

duration of the third phase We have decided to use the velocity at 200 ms after release in plots of the force—velocity relationship The disadvantage of using a fixed time point for this purpose is that the measurements will be taken at somewhat different lengths of the contractile element when the muscle shortens against a high and a low load respectively Shortening velocity could have been measured at a defined length of the contractile element if the end point of the series elastic recoil (phase 1) could have been determined with accuracy This is difficult due to the transient oscillations and furthermore the measuring points with this approach might have become widely separated in time Standardizing the velocity data on the basis of a fixed time point after the release therefore seems preferable

Fig 5 summarizes the force—velocity relations obtained for contractures elicited by 2 5 and 0 4 mM Ca respectively The isometric contractile force was on the average, 1 7 times higher when the muscles were contracted by 2 5 mM Ca than when 0 4 mM Ca was used To permit comparison between the two sets of data the load has been given on the abscissa as a fraction of the total isometric force P_o The results of these experiments as summarized in Fig 5 indicate that the rates of shortening at the different levels of relative load P/P_o are independent of the [Ca++] used to initiate the contracture

Discussion

The present method of producing contracture responses by addition of Ca++ to an already depolarized and Ca depleated muscle has certain important advantages for a study of this kind It implies that the contractile response is indeed dependent on the supply of Ca from the extracellular source and also that the contracture is not

preceded by spike induced twitches (*cf* Biamino and Johansson 1970) As the contracture tension was always measured at its sustained plateau it can be considered to represent an equilibrium condition with regard to the influence of Ca^{++} As judged from the experiments reported in section I above the contractile force of the depolarized portal vein is determined by a rate limiting process in which two calcium ions react with one molecule of the "receptor compound If the linearity of the reciprocal plot of response versus $[Ca^{++}]^{2}$ is not merely fortuitous it reflects the function of Ca at some crucial site in the tissue The cellular localization of the unknown receptor substance can only be a matter of speculation at this stage but two possibilities seem to deserve special consideration The receptor compound could be involved in the transport of Ca^{++} into the cell thereby determining the level of $[Ca^{++}]$ which in turn would directly regulate the contractile force Alternatively the receptor sites reacting with two calcium ions could be located in the contractile apparatus itself

It may be of interest to compare the present results concerning isometric force as a function of $[Ca^{++}]$ with observations made on other contractile systems In a recent study of contracture responses in frog atrium Chapman and Tunstall (1971) found tension to be related to a cubic function of $[Ca^{++}]$ An initial twitch response was evident in many of their mechanical recordings and it seems likely that the cellular mode of activation in their study might have differed from that in the contractures of the present experiments Studies on intestinal smooth muscle reported by Hurwitz and Suria (1971) in a recent review indicated a direct linear relationship between $[Ca^{++}]$ and tension The experimental procedures were not described in detail and comparison with the present study is difficult

Certain observations which refer directly to contractile proteins are of interest with regard to the cellular localization of the hypothetical receptor compound responsible for the relation of tension to $[Ca^{++}]^2$ in portal vein In studies of barnacle muscle by means of the aequorin technique, Ashley (1970) found tension to be related to $[Ca^{++}]_i^2$ Tension development in glycerinated vascular smooth muscle (Filo, Bohr and Ruegg 1965) and ATPase activity of vascular actomyosin (Sparrow *et al* 1970) show steep curves for their Ca dependence and these curves might reflect a square function Such observations may suggest that the relation between contracture tension and $[Ca^{++}]^2$ in portal vein could be due to an effect of Ca in the contractile apparatus itself despite the fact that the Ca sensitivity of the proteins refers to concentrations which are about three orders of magnitude below the extracellular levels involved in the present study However as pointed out above we have at present no experimental indications as to the cellular localization of the mechanism responsible for the correlation between contractile force and $[Ca^{++}]_o^2$

Whereas the isometric force of the depolarized portal vein is clearly dependent on Ca^{++}, it appears from the experiments of section 2 above as if the velocity of shortening at any given relative load may be independent of the $[Ca^{++}]$ used to elicit the contracture Similar results have been obtained on skinned fibres of frog skeletal muscle by Podolsky and Teichholz (1971) Their findings were interpreted in terms

of the sliding filament model (*e g* Huxley 1957) to indicate that [Ca] determines the number of active cross bridges and thereby the level of P while the movement of the individual bridge is only a function of its load and of the inherent characteristics of the muscle as reflected in its V_{max} Relative load (P/P_0) for the entire muscle would then be representative also of the relative load on the individual cross bridge In contrast to Podolsky and Techholtz (1971), Julian (1971) concluded from experiments on chemically skinned fibres that Ca does have an effect on shortening velocity as well as on isometric force The inability of the former investigators to detect this influence was attributed by Julian to deterioration of their preparations and to their small number of observations at low relative loads Studies of twitch responses in cardiac muscle have been interpreted to indicate a rise in V_{max} as well as in P_0 when contractility is enhanced by increased contraction rate (Edman and Nilsson 1969) or by elevation of [Ca] (see *e g* Brutsaert Claes and Sonnenblick 1971)

The contractile effect of changes in extracellular [Ca] in the present experiments on depolarized smooth muscle are assumed to be mediated through variations in the intracellular Ca As the preload often amounted to some 20 per cent of P_0 the very low levels of relative load could not be examined by the quick release method A small or moderate effect of Ca on shortening velocity might therefore have escaped detection but Fig 5 speaks against any pronounced influence of this kind It must be pointed out however that the differences in contractile element length might lead to relatively lower shortening velocities at the high Ca level

Application of Hill s equation (Hill 1938) to the data in Fig 5 would seem to give an approximate V_{max} for the smooth muscle of the portal vein This value would then enable us to assign a place to this preparation in classifications of muscles based on shortening speed (see *e g* Ruegg 1971) However a V_{max} obtained in this way from Fig 5 would have to be judged with caution for several reasons Again the lack of data at very low levels of relative load would make the extrapolation uncertain It must also be remembered that the shortening speeds obtained in the present study refer exclusively to the situation at 200 ms after release The maximal rates of shortening of the contractile element may be higher at an earlier point and a value of V_{max} obtained from Fig 5 would then be too low A further improvement of the isotonic recording system in order to reduce the inertial oscillations may give us more reliable information on shortening velocities in the high range

The recent demonstrations of both thick and thin myofilaments in smooth muscle (Devine and Somlyo 1971 Garamvolgyi Vizi and Knoll 1971 Rice *et al* 1971) give morpohological background for a sliding filament theory of contraction in this tissue as well as in striated muscle It is possible therefore that further investigations on the mechanics of smooth muscle may contribute to our understanding of contraction in general

This study was supported by grants from the Swedish Medical Research Council (B72 28 14X 08C) and from AB Hassle The able technical assistance of Mrs Monica Lundahl is gratefully acknowledged

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Acta physiol. scand. 1972. 84. 538—542
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Catecholamine Storing Cells in Human Fetal Superior Cervical Ganglion

By

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Received 21 October 1971

Abstract

Hervonen, A. and L. Kanerva. *Catecholamine storing cells in human fetal superior cervical ganglion.* Acta physiol. scand. 1972. *84.* 538—542.

The distribution and structure of small, intensively fluorescent, granule containing cells within the human fetal ganglion cervicale superius was studied. The ganglia contained numerous small collections of these cells evenly scattered over the whole region of the organ. The granule-containing cells were as a rule located on the capillaries and showed regularly tight contacts with the basement membrane. The mean diameter of the catecholamine storing granules was 2700 Å. The differentiation and role of the granule-containing cells within sympathetic nervous system was discussed.

After the first discovery of "small intensively fluorescent cells" (SIF) in the superior cervical ganglion of the rat (Eränkö and Härkönen 1963, 1965) several authors have reported the presence of a catecholamine storing cell type within sympathetic ganglia of various mammalians (Elfvin 1968, Siegrist *et al.* 1968, Matthews and Raisman 1969, Williams and Palay 1969, Eränkö and Eränkö 1971, Karnева 1971, Watanabe 1971 and others). The discovery of efferent synapses formed by the SIF-cells (Matthews and Raisman 1969) led to the hypothesis of an autonomic inter neuron. The other possible functions of the SIF-cells were reviewed recently by Eränkö and Eränkö (1971).

The present investigation was undertaken to study the characteristics of developing catecholamines storing cells (CSC cells) within the sympathetic nervous system of human fetus.

Material and methods

The preliminary material consisted of 7 human fetuses aged 13—17 weeks. The fetuses were ... ...tomy. The perfusion fixa... ...circulation and lasted for ... fixative for an additional ... de in 0.075 M phosphate buffer 0.3 M glucose ... ...way and were post stained

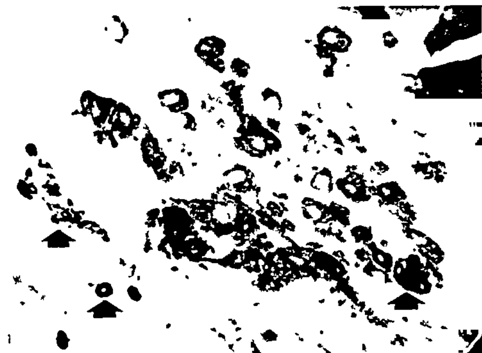


Fig 1 Formaldehyde induced catecholamine fluorescence in the superior cervical ganglion of 18 weeks-old human fetus Collections of SIF cells (catecholamine storing cells) are pointed out by arrows A few of the sympathetic neuroblasts also exhibit intensive yellow fluorescence 600 ×

with both uranyl acetate and lead citrate Philips EM 300 was used in studying and photographing the specimens

For fluorescence microscopy the superior cervical ganglia were prepared and freeze dried following the general rules presented by Eranko (1967) and Olsson and Ungerstedt (1970) The gangl[illegible] embedded [illegible] —5 μ sections were cu[illegible] rtholux microscope e[illegible] ere used One BG 38 [illegible] ters by Schott) and K[illegible] by Ploem

Results

The superior cervical ganglia of a midterm human fetus contains primitive sympathetic nerve cells representing almost the whole scale of differentiation from the primitive sympathetic cell to morphologically mature neuron with synaptic profiles on their surface The development of the sympathetic neurons will be described elsewhere (Hervonen and Kanerva 1972)

Small intensively fluorescent cells (SIF cells) were present in every ganglion processed for fluorescence microscopy The primitive ganglion cells already exhibited weak green fluorescence in the youngest fetus of the present material The small in

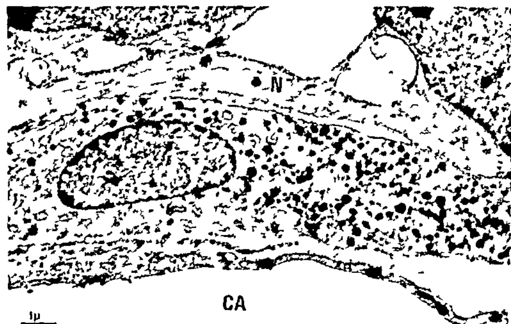


Fig 2 Catecholamine storing cell in a superior cervical ganglion of 16 weeks-old human fetus The cytoplasm is packed with large (mean diameter about 2700 Å) catecholamine storing granules with very electron dense and homogenous core The cell is in close contact with the basement membrane of the capillary (CA) and neuronal processes are found on the plasma membrane (N 14 600 ×

tensively yellow fluorescent cells were evenly scattered over the whole area of the ganglion The solitary cells were often strongly spindle shaped and seemed to be in close contact with the local capillaries (Fig 2)

The fine structure of the cells resembled the structure of catecholamine storing cells CSC in human fetal paraganglia and adrenal medulla to a great extent (Hervonen 1971) The CSC were found only in tight contact with the basement membrane of the capillaries Also if groups of catecholamine storing cells were found capillaries invariably tangentiated the group The solitary catecholamine storing cells on the capillaries were elongated and faced the capillary wall tightly The plasma membrane was mostly covered with neuronal processes from the primitive neurons and/or preganglionic nerves Although synapses were found on the maturing neurons they were not met on the surface of CSC Only the noradrenaline type of catecholamine storing granules was observed in the cytoplasm of CSC The mean diameter of the granules was 2700 Å and their shape was irregular, very elongated forms were also present in addition to the round and ovoid ones

Discussion

Considerable amounts of catecholamine storing small intensively fluorescing cells storing catecholamine were found within the superior cervical ganglia of the mid-term human fetus

The CSC (or SIF) cells were always in close contact with the capillaries of the ganglion The localization of certain endocrine celltype of capillaries would naturally provide good conditions for the release of substances into the circulation. The possibility of the endocrine nature of SIF cells has been discussed by several authors (for ref see Eränkö and Eränkö 1971) However, during the ontogenesis, other explanations for the perivascular localization are also conceivable

The origin of these catecholamine storing cells within the sympathetic ganglia has not been studied before Lempinen (1964), however, demonstrated that the amount of chromaffin cells in rat sympathetic ganglia increased after injections of hydrocortisone in newborn rats He suggested that the hormone stimulated the differentiation of catecholamine containing cells in the ganglia The organization of the newly differentiated cells was random Recently Eränkö and Eränko (1972) confirmed the results by means of fluorescence microscopy They found that the amount of small intensively fluorescent cells was increased tremendously in the superior cervical ganglia of the hydrocortisone handled rat A similar effect was also observed in other ganglia of the sympathetic thrunk Both these findings indicate that the amount of SIF-cells within the sympathetic ganglia may be widely dependent on the plasma levels of some circulating humoral agents

More evidence on possible humoral guidance of the differentiation of CA storing cells was reported recently by Hervonen (1971) who found that the further differentiation of primitive sympathetic cells which serve as a common origin for both sympathetic neurons and catecholamine storing cells might be dependent on whether or not the cells possessed good microcirculatory contacts The differentiating CA storing cells were always found in good contact with the primitive microcirculation whereas the developing neurons often lack this kind of intimate vascular contacts

It seems to the authors that the perivascular catecholamine containing cells of the superior cervical ganglion are the result of a misdirectioning of the differentation caused by the circulating steroid hormones Instead of differentiating into sympathetic neurons a small proportion of the primitive sympathetic cells assumes the enzyme patterns of catecholamine storing cell type Close contacts between SIF cells and the local capillaries has been reported by several authors in various mammalian ganglia (Siegrist *et al* 1968 Matthews and Raisman 1969 Eranko and Eranko 1972 Kanerva 1972, Kanerva and Teravainen, 1972)

Synapses were not found on the catecholamine storing cells which does not exclude the possibility that they may develop later during the organogenesis period To summarize, the catecholamine storing cells seemed to develop randomly within the sympathetic nervous tissue only where sufficient circulatory contacts were present Whether or not these cells are of functional importance for the ganglionic transmission remains an open question

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Acta physiol. scand. 1972. 84. 543—549

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Effect of Physical Activity on the Metabolism of Collagen in Aged Mice

By

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Abstract

HEIKKINEN, E and I VUORI *Effect of physical activity on the metabolism of collagen in aged mice* Acta physiol scand 1972 *84* 543—549

Male mice 12—14 months old [illegible] of 4 weeks Another [illegible] considerably limited [illegible] ated proline was inje[illegible] where upon the turnover of collagen and other proteins was measured and the contents of hexosamine, hydroxyproline, nitrogen, calcium, potassium and sodium were determined in various tissues Metabolism of collagen was increased by exercise and decreased by physical inactivity especially in Achilles tendon [illegible] calcium, potassium and sodium ions in long bones and in the heart and skeletal muscles were observed The results show that the metabolism of connective tissues is influenced by degree of physical activity even in relatively old animals

Metabolism of connective tissue decreases with the age of the organism, and accompanying structural changes lead to increased dehydration and crystallinity (*cf* Hall 1969) Changes both in metabolism and structure occur faster during growth and development, in an adult organism metabolism of collagen is slow (*cf* Heikkinen 1969) and is not known to alter considerably under physiological conditions Physical training increases the number of cells and the contents of collagen and ground substance in the articular cartilage of rabbits and thickens ligaments and tendons (Ingelmark 1957) Running causes muscle and bone hypertrophy in the rat (Saville and Whyte 1969) and strengthens knee ligaments in rats (Tipton 1967) but the density of bones does not change On the other hand, immobilization causes negative calcium balance and osteoporosis in bones (*cf* Jowsey 1969) in which metabolism of the organic matrix is impaired Very little is known, however, of the

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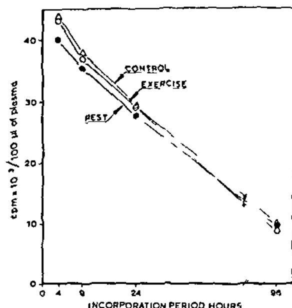


Fig 1 Total radioactivities of 100 µl of blood plasma 4—96 h after intraperitoneal injection of tritiated proline (1 µCi/g) in the exercised inactive and control mice Points represent the means of 5—6 determinations

effects of physical activity on the metabolism of collagen This paper shows that exercise increases and physical inactivity decreases the metabolism of collagen as well as that of other components of several connective tissues in aged mice To our knowledge no general analysis of this nature has been reported earlier Preliminary data of the investigation have been published elsewhere (Heikkinen and Vuori 1970)

Material and Methods

Animals and experimental conditions

White male mice of NMRI strain (Yla Mankkaan tila Mankkaa Finland) aged 12—14 months were used The mice were divided into 3 groups Group 1 (Exercise) was trained on a treadmill daily for an average of 4 weeks at a speed of 30 cm/s The time of the training sessions was increased gradually to [illegible]
fourth of the volume of the normal [illegible]
(Control) served as a control an[illegible]
mice diet (Hankkija Finland) was [illegible]
ginning of the experiment was 34 35 g In the exercised group the average weight decreased 2—3 g during the experimental period In the other groups the average weights remained unchanged

Incorporation of radioactive proline

Radioactive proline (TRA 82 Radiochemical Centre Amersham England) 1 µCi/g was injected i p at the end of the experimental period Incorporation was allowed to continue for 4, 9, 24 and 96 h Altogether 9 mice from all three groups were killed after each incorporation period Training and limitation of normal motility were continued during the incorporation period

Preparation of tissue samples

A sample of *blood* was taken after thoracotomy by open heart puncture and centrifuged for 15 min at 3000 rpm. 100 µl of plasma was transferred to the counting vial for the determina

tion on radioactivity Fig 1 shows the levels of plasma radioactivity in the three groups of mice after each incorporation period

Skin was removed and hair and subcutaneous tissue were removed The cleaned skin was cut to pieces with scissors and weighed samples of the pieces were used for determinations

The *heart* was dissected, opened, immersed several times in 0 9 % NaCl to remove blood,

soleus were dis-
r the determina-
other leg were
eterminations

The *humerus* and *femur* were separated, muscle and loose connective tissue were removed and the bones weighed. One humerus and one femur were used for the determination of radioactivity, metals, hydroxyproline and nitrogen Hexosamine was analyzed from the other pair of bones

Chemical analyses

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...by Woessner (1961) The concentration of *nitrogen* was measured after combustion of the sample ... (1963)
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For the determination of *hexosamine*, tissue samples were hydrolyzed in 2 N HCl for 16 h at 103° C The hexosamines were freed from interfering chromogens by means of Dowex-50 cation exchange resin according to Boas (1953) and the colour was developed by Blix's (1948) modification of the Elson Morgan method

Calcium, potassium and *sodium* were determined with an atomic absorption spectrophotometer (Unicam SP-90, Unicam Instrument Ltd , Cambridge, England)

Statistical significances of the differences between the three groups of mice were calculated using a modification of Student's *t* test (paired comparison, Hill 1967)

Results

Achilles tendons

Total radioactivities (Fig 2) and specific radioactivities of hydroxyproline (Fig 3) indicate that the turnover of collagen and other proteins in achilles tendons was increased by running and decreased by physical inactivity The ratio of hydroxy proline to nitrogen in the Achilles tendons was highest in the exercised mice (Table I) Quantitative amounts of collagen were not obtained because of difficulties in total dissection of the tendons

Humerus and femur

Increase in the turnover rate of total proteins (Fig 4) and of collagen (Fig 5) was observed in long bones of the trained mice Physical inactivity on the other hand, retarded protein and collagen metabolism The contents of hydroxyproline and nitrogen were about the same in all three groups but the content of hexosamine was slightly increased by exercise and decreased by physical inactivity (Table I).

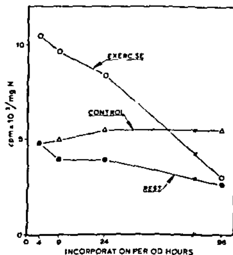


Fig 2

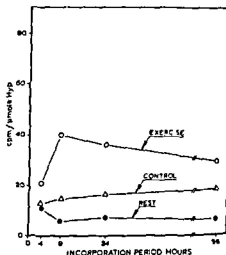


Fig 3

Fig 2 Total radioactivities of Achilles tendons after injection of tritiated proline Points represent the means of 7—9 determinations

Fig 3 Specific radioactivities of hydroxyproline in Achilles tendons after injection of tritiated proline Points represent the means of 3 determinations

Heart and skeletal muscles

Total radioactivity of heart muscle decreased from about 10,500 cpm/mg of nitrogen 5500 cpm/mg of nitrogen during the incorporation period The corresponding radioactivities of skeletal muscles of hind legs were 4000 cpm/mg of nitrogen and 2000 cpm/mg of nitrogen A slight increase in collagen metabolism in heart muscles of the exercised group and a decrease in the inactive group was observed (Fig 6) Specific radioactivities of hydroxyproline in skeletal muscles were too low to be measured reliably

TABLE I The ratios of hydroxyproline to nitrogen in Achilles tendons and contents of hexosamine hydroxyproline and nitrogen in humerus and femur of the exercised inactive and control mice

Mice	Achilles tendon		Humerus and femur	
	Hypro/N	Hexosamine (µg)	Hydroxyproline (mg)	Nitrogen (mg)
Trained	63 ± 04(8)	115 1 ± 3 4(8)	2 33 ± 14(8)	4 80 ± 18(8)
Inactive	57 ± 03(8)	107 6 ± 4 3(8)	2 32 ± 16(8)	4 73 ± 26(8)
Control	54 ± 03(8)	110 5 ± 4 9(8)	2 23 ± 09(8)	4 42 ± 28(8)

Note — The means, their standard errors and the number of determinations are given p-values of the differences in the hydroxoproline/nitrogen ratios were < 01 between exercised and inactive mice and < 02 between trained and control mice

Corresponding p values of the differences in the hexosamine contents were < 01 and < 02

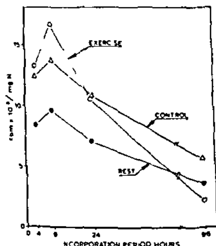


Fig 4

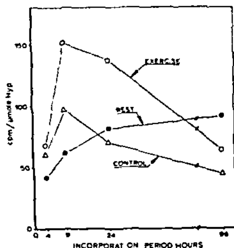


Fig 5

Fig 4 Total radioactivities of humerus and femur after injection of tritiated proline Points in this and in the following figures represent the means of 7—9 determinations

Fig 5 Specific radioactivities of hydroxyproline after injection of tritiated proline in the humerus and femur of the exercised inactive and control mice

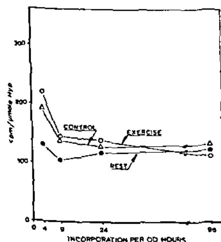


Fig 6

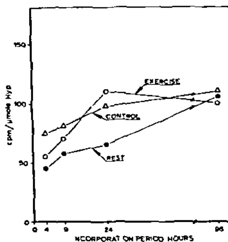


Fig 7

Fig 6 Specific radioactivities of hydroxyproline in heart muscle after injection of tritiated proline in the exercised inactive and control mice

Fig 7 Specific radioactivitiets of hydroxyproline in skin after injection of tritiated proline in the exercised inactive and control mice

Skin

Slight retardation in the metabolism of collagen was observed in the skin of inactive mice (Fig 7) The contents of hexosamine and nitrogen (µg/mg of wet weight) were highest in the skin of the exercised mice (Table II)

TABLE II Contents of hexosamine and nitrogen in the skin of the exercised, inactive and control mice

Mice	Hexosamine (µg/mg of wet weight)	Nitrogen (µg/mg of wet weight)
Trained	73 ± 02(8)	46 5 ± 2 4(8)
Inactive	66 ± 03(8)	42 0 ± 2 1(8)
Control	66 ± 02(8)	42 2 ± 2 3(8)

Note — The means, their standard errors and the number of determinations are given p-values of the differences in the contents of hexosamine and nitrogen were < 05 between exercised and control mice and < 02 between exercised and inactive mice

Discussion

The results show that even in relatively old animals the degree of physical activity influences the metabolism and structure of connective tissues This effect is seen especially in Achilles tendons (Fig 2—3, Table I) and in weight bearing long bones (Fig 4—5, Table II) *Exercise increases the relative amount of collagen in Achilles* tendons and accelerates the turnover of collagen and other proteins while physical inactivity has an opposite effect Similar changes in protein and collagen metabolism is observed also in long bones although no hypertrophy was found contrary to the observations of Saville and Whyte (1969), who used one month old rats These reactions can be regarded as significant adaptation responses by the organism to physical stress Possible effects of the length of the training period on collagen metabolism remain to be studied Our preliminary data on the amounts of RNA and DNA show that the number of cells in bones is not altered in aged animals by the training program used This suggests that the observed increase in the synthesis and turnover of proteins is not the result of cellular hypertrophy but is mainly based on stimulation of the metabolism of existing cells Reasons for this stimulation need further clarification as does the effect of age on the responses of connective tissues to exercise Increased oxygen supply or utilization and certain hormonal effects should be considered the basic mechanisms of the enhanced metabolism

Financial aid from Suomen Kulttuurirahasto from Research Council for Medical Sciences (Finland) and from Research Council for Physical Education and Sports (Finland) is gratefully acknowledged

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W[illegible] l protein samples [illegible] 440—447

Acta physiol. scand. 1972. 84. 550—554

From the Department of Medical Chemistry, University of Turku, Finland

Assay of the Transformation of Collagen in situ

By

E. HEIKKINEN, K. IVASKA and E. KULONEN

Received 21 October 1971

Abstract

HEIKKINEN E., K. IVASKA and E. KULONEN *Assay of the transformation of collagen in situ*. Acta physiol. scand. 1972. 84. 550—554.

Skin extract of newborn rats increases the insolubilization of collagen in skin slices *in vitro*. *The effect is destroyed by* heating and inhibited by lathyrogens. Purified collagen in the precipitated form is, on the contrary, transformed to the more soluble forms by skin extract.

The physiological importance and the metabolic stability of collagen depend on the solid phase but the solubility and mechanical strength do not necessarily parallel. The insolubility is due to the close packing but mainly to the cross-linking of tropocollagens either by covalent cross links between tropocollagens or through some intermediary substances, for example glycoproteins. The chemistry and biochemistry of intermolecular cross-links have been advanced recently through the work from the laboratories of Bailey (Bailey 1968, Bailey and Fowler 1969, Bailey *et al.* 1970), Martin (Pinnell and Martin 1968, Siegel and Martin 1970a, Siegel *et al.* 1970b) and Nimni (Desmukh and Nimni 1971). Various cross links are produced between lysines (or hydroxylysines) and their carbonyl derivatives. It remains to be studied what are the limiting factors in the tissues and whether accessory substances contribute to the formation of the network. Therefore we have designed the present system to assess the insolubilization directly.

The preparations and the procedure are described in Fig. 1.

The insoluble fraction of collagen increased when skin slices were incubated with skin extracts as seen in the changes of the distribution of the total radioactivity between the various collagen fractions (Table I). The effect of skin extract is destroyed by heating. Lathyrogens (aminoacetonitrile and semicarbazide) in millimolar concentrations have an inhibitory effect on the insolubilization. The insolubilization of collagen during the incubation is continued at least for 16 h (Fig. 2). The extract is active at pH 7.0 but not at pH 6.0 or 8.0 (Fig. 3).

Fig 1 Flow sheet on the insolubilization of collagen *in vitro*

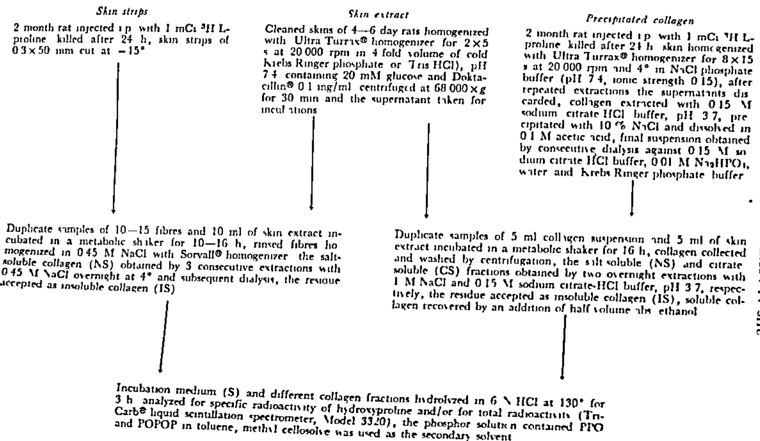


TABLE I Effect of skin supernatant on the insolubilization of collagen

Incubation medium	Incubation temperature	Distribution of radioactivity of the collagen fractions					
		S %	NS %		IS %	IS/NS	Ratio of spec. activities of hydroxyproline in IS and NS-fractions
Skin strips							
Krebs Ringer phosphate alone	37°	18	36		46	1 3	1 2
		19	25		56	2 2	1 8
Skin extract	±0°	33	48		19	0 4	0 2
		27	54		19	0 4	0 2
Skin extract	37°	22	13		65	5 0	4 0
		21	8		71	8 9	8 1
Heated skin extract (5 min above 80°)	37°	26	30		44	1 5	1 2
		26	26		48	1 9	3 5
Tris HCl	37°	14	67		19	0 3	0 2
Skin extract	±0°	3	81		16	0 2	0 2
Skin extract	37°	27	31		42	1 4	2 3
+0 25 mM aminoacetonitrile		14	57		29	0 5	0 5
+2 5 mM aminoacetonitrile		11	53		28	0 5	0 4
+0 25 mM semicarbazide		9	68		29	0 4	0 2
		S %	NS %	CS %	IS %	IS/(NS+CS)	CS/NS
recipited collagen							
Krebs Ringer phosphate alone	30°	3 1	3 1	50 6	43 2	0 8	16 3
Krebs-Ringer phosphate alone	±0°	3 1	2 3	53 1	41 5	0 8	23 1
		3 9	2 3	51 4	42 4	0 8	22 3
Skin extract	30°	17 1	19 2	26 1	27 6	0 5	0 9
		16 7	31 1	22 8	28 4	0 5	0 7
Heated skin extract (5 min above 80°)	30°	12 1	49 7	6 3	31 9	0 6	0 1
		12 8	39 9	11 8	35 5	0 7	0 3

The abbreviations explained in Fig 1

When precipitated collagen is treated similarly at 30°, there is no increase in the insoluble fraction but on the contrary a shift to the more soluble forms (Table I)

The formation of aldehydes presumably through the deamination of lysines seems to be the main enzymological event but it is not clear whether lysine amino oxidase acts alone, how the function of the enzyme(s) is modified by the conditions in the tissue, and whether also non collagenous components are involved A pre-treatment of the tissue substrate to break the various components should give a clue The acidic structural proteins, glycoproteins and acidic proteoglycans should be especially considered Similar results on precipitated collagen were obtained by

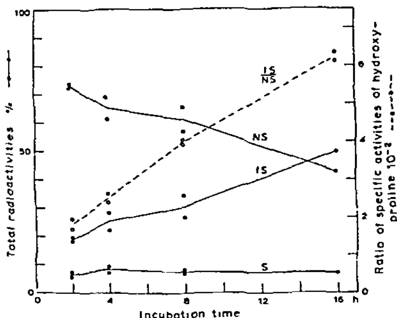


Fig 2 *Formation of insoluble collagen in skin slices in vitro at the incubation with a skin extract from 4—6 day rat.* IS insoluble collagen NS neutral salt-soluble collagen, S incubation medium

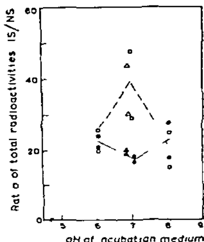


Fig 3 Effect of pH on the formation of insoluble collagen *in vitro* O——O with skin extract ● ● without skin extract in 0 1 M citrate phosphate buffers △ ▲ in Krebs-Ringer phosphate respectively

Majaniemi and Kulonen (1964) In tissues there seems to be a relatively heat stable collagen labilizing or collagenolytic factor (*cf* Rabadjija *et al* 1971) Kuhn *et al* (1964) observed that purified tropocollagen does not aggregate so well as less purified samples

Institutional *support from* U S *Department of Agriculture Foreign Research* and Technical Programs Division and from the Sigrid Juselius *Foundation is gratefully acknowledged*

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From the Department of Physiology, University of Umeå, Sweden

Properties of Cortical Group I Neurones Located in the Lower Bank of the Anterior Suprasylvian Sulcus of the Cat

By

H. SILFVENIUS

Received 22 October 1971

Abstract

SILFVENIUS, H. *Properties of cortical group I neurones in the lower bank of the anterior suprasylvian sulcus of the cat.* Acta physiol. scand. 1972. *84*. 555—576.

Record [illegible] of the [illegible] regard [illegible] latera [illegible] affere [illegible] affere [illegible] thresh [illegible] Foreli [illegible] types [illegible] *cal gr* [illegible] *I* cell [illegible] by a [illegible] from [illegible] they [illegible] with [illegible] ceived excitation from other afferents or did not respond to the inputs used. The results are discussed in relation to previous studies on forelimb group I projections.

Focal potential studies have shown that group I muscle afferents from the forelimb influence neurones in the contralateral cerebral cortex of the cat in the region of the postcruciate dimple, Pcd, (Oscarsson and Rosén 1963), in the hidden lower bank of the anterior suprasylvian sulcus, ASSS (Landgren, Silfvenius and Wolsk 1967 a), in the rostral bank of the lateral ansate sulcus and in the lateral sigmoid gyrus (Silfvenius 1968). Afferent connexions to single group I influenced cells in the Pcd have been studied by Oscarsson, Rosén and Sulg (1966), Swett and Bourassa (1967) and by Grampp and Oscarsson (1968). Monosynaptic excitation by thalamocortical axons of the forelimb group I path was established. Excitation as well as inhibition from low threshold forelimb skin afferents in the same cells was of longer latency and the inhibition evoked by group I afferents in other cells was disynaptically linked to thalamocortical axons.

The focal potential study of the group I area in ASSS also revealed projection from group I and low threshold skin afferents (Landgren *et al* 1967 a) Convergence from auditory and vestibular afferents was seen in the nearby and overlapping parts of the lower bank of ASSS (Landgren, Silfvenius and Wolsk 1967 b) An understanding of the projection patterns in the group I locus of ASSS does however, require single unit analysis The results of such an investigation are presented in this report

Methods

Anesthesia and preparation 22 cats were used The anesthesia was induced with 2 % Halothan (Hoechst) vaporized into a 2 1 mixture of O_2—N_2O and continued with chloralose 70 mg/kg i v Before unitary recordings the animals were paralyzed with Flaxedil (May and Baker) and artificially ventilated with a 96 % O_2—4 % CO_2 mixture The effect of Flaxedil was allowed to wear off at times and the depth of anesthesia and the adequacy of ventilation were checked Blood pressure, body temperature and temperatures of the paraffin pools covering the exposed tissue were monitored The body temperature was kept around 37° C with a heating lamp The animals were infused with Aminosol glucose (Vitrum) Ventol (Knoll) was given intravenously when required in a 1 20 solution to prevent fall in blood pressure

... ned for electr ... erve ... the ... ected through a medial incision Ejt was identified according to the method described by Andersen *et al* (1967) and pulled together with Bic through a slit to the extensor side The medial wound was sutured and sealed with collodion and a pool was constructed on the extensor side The nerves distal to Bic were cut The afferent volleys of Bic Tri DR and SR were recorded from the surface of the dorsal column at C_4 s level The afferent volley of Ejt was recorded from the musculocutaneous nerve

In the right hind limb the following nerves were dissected the nerves to the posterior biceps-mitendinosus muscles (PBSt) the nerves to the gastrocnemius-soleus muscles (G S) the sural nerves (Su) and the posterior knee joint nerve (Kjt) The other nerves of the hind limb were cut except those of its medial side The afferent volleys of PBSt G S and Su were recorded from the sciatic nerve that of the Kjt from the tibial nerve

In the left cerebral hemisphere the cortex of the postsigmoid anterior suprasylvian and ectosylvian areas was exposed The expression contralateral refers to the cerebral hemisphere

A cervical laminectomy was made at C_4 $_6$ levels and the spinal cord was exposed

Methods of recording The animal was placed in a stereotaxic apparatus with the head in normal position Unitary recordings were performed in a closed chamber made of perspex, which was sealed to the cranium with Dental Kerr Compound On the chamber was mounted a circular perspex disc attached to the micromanipulator The disc was levelled in the horizontal Horsley Clarke plane In the center of the disc was a hole through which a Delrin tube was pressed The inner diameter of the tube matched the outer one of the steel tube into which the glass pipettes were baked with dental cement The top of the chamber contained a rubber ... The disc with the micromanipulator could ... opened and the pipette introduced the ... wall This closed system reduced move- ... -citrate had resistances of about 5—15 ... optical control usually through a small ... e connected to the CRO via a cathode ... ncy band width (down to 3 db) of 10 Hz—5 kHz The resting membrane potential was recorded by connecting the cathode follower directly to the CRO The responses were recorded as photographic superpositions of multiple sweeps or as single sweeps

Methods of stimulation The electrical stimuli to the dissected nerves were condenser discharges delivering pulses of 100 μs Visual stimulation was given as single 3 ms flashes of white light to the atropinized right eye via a perspex rod placed close to the eye Auditory stimulation was a free field click from a loudspeaker placed about 10 cm from the left ear The click was produced by feeding a 100 μs pulse into the loudspeaker The stimulus strengths are expressed in multiples of the intensity (T) necessary to evoke a discernible potential in primary

afferents The thresholds of the cortical unitary potentials were related to T At times, responses were evoked in cells at such a low stimulation intensity that no volleys were discernible in primary afferents The thresholds of such cells were therefore considered less than 1 0 T The monitor of the afferent volley was placed on the spinal cord (C_4—C_5) in a position chosen to give maximal amplitude of the group I spike In some experiments occasional low threshold axons escaped detection by this method The cases with thresholds below 1 0 T were however few (*cf* Fig 3) and the conclusions drawn from the observations are not affected by a [illegible]

[illegible] ned to evoke cortical potentials [illegible] ty primary spindle afferents are [illegible] (Laporte and Bessou 1957), a [illegible] and some joint afferents Group II muscle afferents are considered activated at strengths between maximal group I and 10 T (Eccles and Lundberg 1959) Low threshold skin afferents are arbitrarily defined as those activated at stimulation strength ⩽1 5 T [illegible] strengths Low threshold joi[illegible] 1967) and high threshold on[illegible] tials evoked by group III m[illegible]

Results

1) *Description of cell sample*

This study describes responses of single cortical units located in the group I locus of ASSS This was defined by Landgren *et al* (1967 a) as the area of the lower bank of ASSS where a focal potential is evoked by group I muscle afferents from the contralateral forelimb at a threshold of 1 0 T Recordings were made from 134 single neurones of this locus 62 (46 %) of them were excited by forelimb group I afferents and 9 (7 %) received inhibition or mixed inhibition excitation from these afferents Thus were in all 71 (53 %) of the cells linked to group I forelimb afferents 45 of the group I influenced cells were studied with intracellular (IC) 14 with IC and extracellular (EC) and 12 cells only with EC recording technique

The EC recorded spike potentials were either monophasic negative or diphasic positive-negative The potentials are assumed to have been recorded from cell somata according to the criteria used by Gordon Landgren and Seed (1961) The EC spike discharge could in many cells be studied for more than 1/2 h The IC recordings were in some instances maintained equally long but generally only for a few minutes or less Cells responding with excitatory postsynaptic potentials EPSPs were easier to hold than cells responding with inhibitory postsynaptic potentials IPSPs Many cells with IPSPs were therefore discarded from this report since they did not last long enough to be tested with the inputs used The initial resting membrane potentials reached values up to 50 mV but most cells were studied at lower resting potentials IC recorded action potentials were often observed but the spikes deteriorated rapidly Low amplitude spikes were seen during later stages of IC recordings The EC field potentials were determined after withdrawal of the pipette from the IC position The amplitudes of these potentials were low in comparison with the IC ones

The neurones were analyzed with regard to their connexions with muscle skin and joint afferents in the contralateral limbs and with auditory and visual afferents

Units influenced by group I muscle afferents will in the following be referred to as *group I cells*

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[illegible] activated at stimulation strength [illegible] higher strengths. Low threshold [illegible] *et al.* 1967) and high threshold ones those evoking cortical potentials with thresholds > 2 T. Potentials evoked by group III muscle afferents were not studied systematically.

Results

1) *Description of cell sample*

This study describes responses of single cortical units located in the group I locus of ASSS. This was defined by Landgren *et al.* (1967 a) as the area of the lower bank of ASSS where a focal potential is evoked by group I muscle afferents from the contralateral forelimb at a threshold of 1.0 T. Recordings were made from 134 single neurones of this locus. 62 (46 %) of them were excited by forelimb group I afferents and 9 (7 %) received inhibition or mixed inhibition-excitation from these afferents. Thus were in all 71 (53 %) of the cells linked to group I forelimb afferents. 45 of the group I influenced cells were studied with intracellular (IC), 14 with IC and extracellular (EC) and 12 cells only with EC recording technique.

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The neurones were [illegible] with skin and [illegible] and [illegible]

Units [illegible] group I [illegible] as group I cells.

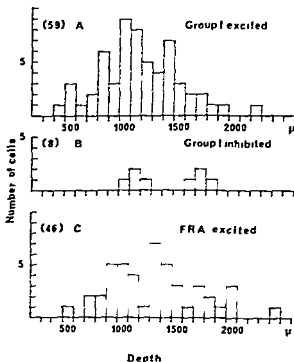


Fig 1 Depth distribution of cells in group I locus of the lower bank of ASSS The depths were determined in relation to the hidden cortical surface in ASSS Cells located medial to the sulcus were excluded (Diagram A 3 cells, B – 1 cell and C = 5 cells) The locations of 2 cells inhibited by FRA are given in the text on p 16 For definition of FRA see text p 13

2) *Location of the main groups of cells*

The lower bank units were localized according to the method described by Landgren *et al* (1967 a) The cortical focal potential evoked by SR was recorded along tracks penetrating through the upper bank across the buried suprasylvian sulcus into the lower bank A polarity shift from an initially negative to a positive potential was observed at the level of layer II in the upper bank and again from positivity to negativity at the corresponding layer of the lower bank The half distance between these polarity shifts corresponded to the hidden surface of the sulcus (*cf* Landgren *et al* 1967 a Fig 1 The lower bank units were localized in relation to the hidden cortical surface and their positions are given in microns below the half distance between the polarity shifts In medial tracks passing more or less tangentially to the cortical layers and therefore lacking polarity shifts the positions of the shifts in near by more lateral tracks were used as reference

The depths of the cells were determined from vertical tracks penetrating the lower cortical bank of ASSS in about 25 degrees angle to the perpendicular columnar orientation of the cortical cells (*cf* Landgren *et al* 1967 a Fig 1 C The determinations do however not introduce greater inaccuracy than 10 % and these errors are considered negligible. No histological localizations of the cells were made in the present series

The distributions of the group I cells within the group I locus are shown in Fig 1 The group I excited cells were found between 480 and 2250 μ below the hidden surface of the lower bank (Fig 1 A) These depths correspond approximately to cortical layers III—VI The group I inhibited cells were located between 1050 and

1800 μ below the surface, thus in the deeper layers V and VI of the cortex (Fig 1 B) No apparent difference in location was observed between group I cells of short and long latency The cells not influenced by group I afferents were located at depths between 590 and 2430 μ (Fig 1 C) There was thus no significant difference in cortical location between the last mentioned cells and the group I excited cells

3) *Properties of group I excited cells*

a) *Discharge patterns* Volleys in forelimb group I afferents influenced the cortical cells differently Propagated action potentials were evoked in about 60 % of the neurones The other responded only with EPSPs Many cells responded with two spikes Cells discharging only one spike, or between three and six spikes, were in the minority The group I cells of the ASSS locus therefore have a discharge pattern similar to that of the group I cells of the Pcd, in which both single and repetitive unitary potentials are recorded (Oscarsson *et al* 1966) The repetitive discharges appeared in some cells already at 1 0 T and often continued unaltered during increasing strengths of stimulation Other cells, however, required summation from a greater number of group I afferents before a constant discharge pattern was established Fig 2 A a—g shows the EC recorded discharge pattern of a cell firing two spikes Stimulation of DR evoked spikes riding on the developing group I focal potential already at threshold strength of stimulation

In IC recordings the spike potentials had amplitudes more than 30 mV in the initial stage of recording Marked afterhyperpolarizations were observed after the spike potentials Later in the recordings the spike mechanism deteriorated leaving only EPSPs or EPSPs with low amplitude spikes Fig 2 B a—g shows the IC records of a group I cell excited by DR In record g the spike is followed by an afterhyperpolarization of 3 5 mV lasting some 40 ms Records a—f taken at faster sweep speed somewhat later show the EPSP evoked by gradually increasing the intensity of the stimulation

The frequency following of group I-EPSPs was tested in some cells and the EPSPs followed 5—20/s repetitions before amplitude reductions occurred

b) *Threshold for excitation* The thresholds of the first three spikes in EC recorded discharges of 16 cells* were determined The first spike appeared at stimulation strengths between 0 9 and 1 5 T mean 1 2 T In about 70 % of these cells a second spike was evoked at strengths between 1 0 and 1 4 T mean 1 1 T A third spike occurred in about 40 % of the cells at thresholds between 1 0 and 1 5 T mean 1 2 T Fig 3 A shows the thresholds of the three first group I evoked spikes—indicated with different symbols—recorded EC in the 16 neurones

The group I cells showed different types of EPSPs Some were simple, often monophasic, composed of a few or several temporally rather closely spaced unitary EPSPs as shown in Fig 2 B Other EPSPs were more complex and appeared to be composed of 2 or more temporally dispersed components of the above mentioned simple

* A sample of 12 EC studied group I cells plus 4 of the corresponding 14 cells studied with EC and IC technique

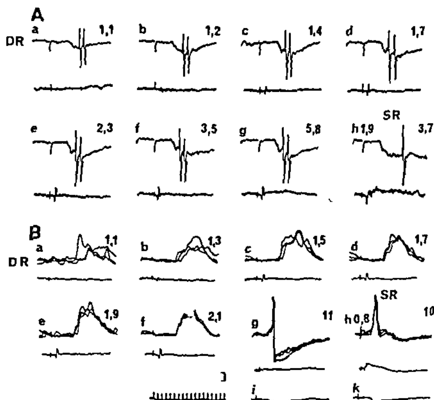


Fig 2 A, upper records extracellular recording from a neurone 1200 μ deep in the group I locus of ASSS discharging action potentials on stimulation of DR group I afferents, a—g, and SR afferents, h B, upper records intracellular potentials of a group I DR excited cell, a—h located at a depth of 1360 μ in the group I locus Resting potential 40 mV Threshold of DR spike potential 1 5 T Records g and h taken before records a—f Co excitation from SR afferents is shown in h Records i and k show extracellular field potentials to stimulations as in g and h Records in A and B under cortical potentials show afferent volleys recorded from the left up- in A mV, g 1 mV, h 2 mV, i—k 4 mV

type (Fig 4 d) In some cells the maximum amplitude of the EPSP was reached with the first, in other with the later components

The thresholds of the simple group I-EPSPs and of the first component of the complex ones were in general similar They were therefore pooled together and their thresholds measured in 50 cells in which group I afferents from different forelimb nerves evoked 65 EPSPs The thresholds ranged from 0 9 to 1 5 T mean 1 1 (cf Fig 3 B). In 24 cells the EPSPs had two or more separable components The thresholds of the second components evoked in 19 cells ranged between 0 9 and 1 5 T mean 1 2 T The thresholds of the first IC recorded spikes varied between 1 0 and 1 5 T, mean 1 2 T, a mean identical with that of the thresholds of the first EC recorded spikes.

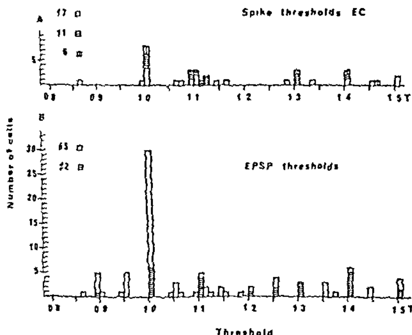


Fig 3 Threshold histograms for extra (A) and intracellularly (B) recorded group I cortical potentials A T of first spike potential (□) in 16 cells with convergence from two nerves in one cell T of 2nd spike (▤) and T of 3rd group I evoked spike (▭) B T of first component of 65 group I EPSPs in 50 cells (□) and T of 2nd component (▤) of 22 EPSPs in 19 cells.

The thresholds of the EPSPs and action potentials of the group I excited cells were thus generally well below 1 5 T This was true also for the second and the third EPSPs and spikes in repetitive discharges The repetitive excitation is therefore due to summation of impulse discharge in low threshold muscle afferents presumably mainly in primary spindle afferents

c) *Time course of EPSPs* The simple type of group I EPSPs (cf Fig 4 a) had a rise time to maximum amplitude (summit time) of 1 2 to 12 0 ms (mean 4 6 ms n = 34) It had a more slowly decaying phase and the total duration was on the average 20 ms (range 10—30 ms) The summit times were measured in two samples of simple group I EPSPs selected for short (≤ 7 ms) and long (> 7 ms latency The short latency neurones (n = 13) had summit times between 1 2 and 8 0 ms mean 3 6 ms SD 2 3 The simple EPSPs (n = 21) of longer latency (cf Fig 4 c had summit times varying between 2 0 and 12 0 ms mean 5 7 ms, SD 2 4 In the complex group I EPSPs the summit times of the first component in short latency responses (n = 19) varied between 1 4 and 5 5 ms mean 3 0 ms SD 1 5 In the long latency complex group I EPSPs the summit times varied between 1 5 and 7 0 ms mean 4 9 ms SD 1 5 The differences of the mean summit times in the two samples of short and long latency cells with simple and complex EPSPs were not

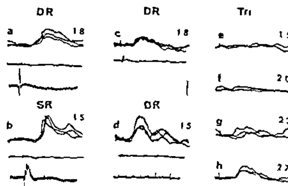


Fig 4 Intracellular potentials from 4 neurones a—b, c, d and e—h located 1020, 1200, 1540 and 1150 μ deep in the group I locus Resting potential for a—b 44 mV, 10 mV for c—h Upper record a shows short- and c long latency simple group I EPSPs. *Record d shows a complex group* I-EPSP Record b shows a short latency SR-EPSP (5 6 ms) evoked with a threshold of 1 0 T. Records e—h show a group II-EPSP evoked by volleys in the Tri nerve (latency 10 ms) Mid records a and b show EC field poten-

shown under records of cortical potentials in a—d, a and b 5 mV, c 1 mV, d 0 4 mV, e—h 1 mV *5 ms for EC field potentials, c—h 5 ms*

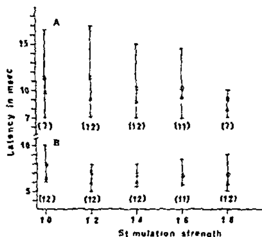
statistically significant Two additional short latency responses, one in each of the simple and complex groups had summit times shorter than 1 ms

The group I-EPSPs reached their amplitude maxima at stimulation strengths between 1 1 and 2 2 T mean 1 4 T These values were obtained from observations in 22 EPSPs of 19 cells in which no contribution from group II muscle afferents was detected and additionally in 12 cells with group II excitatory convergence where the amplitude maxima of the first components were clearly separable from the rest of the combined group I—II responses The amplitudes of the simple EPSPs ranged between 0 2 and 2 5 mV mean 0 7 mV In some cells with maxima reached at 1 8 T the amplitudes were as high as 4 mV These EPSPs were presumably also evoked exclusively by group I afferents

TABLE I Minimum latencies (ms) of excitatory and inhibitory potentials in neurons of the group I locus of ASSS evoked by forelimb muscle and skin afferents

Stimulated afferents	Excitation, type of discharge			Inhibition
	1st component	2nd component	3rd component	1st component
Group I				
EC spike	(16) 6 5—17 0, 11 6	(11) 8 1—21 0, 15 3	(6) 9 5—28 0, 20 4	
IC spike	(29) 5 8—33 0, 12 8	(11) 6 6—19 0, 10 9	(4) 7 4—19 5, 12 3	
IC EPSP	(65) 5 0—15 0, 8 4	(22) 6 6—25 0, 16 4		(10) 6 0—17 0, 9 5
Group II				
EC spike	(17) 6 5—25 0, 16 2			
IC EPSP	(62) 6 0—26 5, 13 1			15) 8 5—22 0, 13 9
Cutaneous				
EC spike	(10) 7 5—19 5, 11 6			
IC EPSP	(50) 5 2—16 0, 8 4			(9) 6 0—13 0, 8 2

Numbers in brackets give number of observations Ranges and means are given



5 Stimulation strength—latency vari e correlation in long (A)—and in rt (B) latency group I cells of ASSS asurements were made on group PSPs in 5 consecutive single sweeps at erent stimulation strengths for each Vertical bars indicate latency range the number of neurones (marked in ckets under bars) tested at each stimu on strength Δ marks the mean of rtest latency and O marks the mean of est latency in the neurone group at erent stimulation strengths (T)

Since the group I cells were depolarized after the impalement the observed ampli les reflect only parts of the values expected in normal cells

1) *Latency* The latencies of the EPSPs and spikes of the group I cells are shown Table I The short latencies (*i e* 5 0—7 0 ms) observed in 22 out of 62 cells, gest that these neurones were monosynaptically activated by thalamocortical group ibres (Oscarsson *et al* 1966 Rosen 1969 b) Other group I cells excited after ger latency could receive their excitation via intracortical linkages Short latency urones might be expected to be more strongly linked to their afferents than long ency cells Neurones excited at short latency by group I afferents would therefore w less variation in latency than those excited at long latency The latency variance 5 consecutive single sweeps was measured at different steps during gradually in asing group I stimulation strength in 12 cells with latencies of 6 ms or less The ults showed that the latency variance in these cells was small throughout the en scale of group I stimulation strength In 12 other cells with longer latencies (7 0 13 5 ms) the latency was greater remaining so at all stimulation intensities These dings are illustrated in Fig 5 where diagram A was computed from cells with ger latency and that of B from cells with short latency A similar pattern of latency riance was observed in the FC material

The narrow latency range in cells with short latency group I responses may imply t these cells have stronger synaptic linkage from group I afferents than cells with ger latencies

Convergence pattern on group I excited cells

Group I afferents 38 (76 %) out of the 50 IC studied group I cells were ex ed by afferents of one forelimb muscle nerve In the EC material of 16 cells it was en more apparent with 94 % of the neurones showing this pattern of excitation nvergence from two muscle nerves was observed in 14 % and from three nerv

TABLE II Convergence of group I excitation on cortical cells of the group I locus of ASSS The patterns are based on extra- as well as on intracellular responses

Number of cells		Forelimb			Hind limb	
		DR	Tri	Bic	G—S	PBSt
48	50	+	—	—	—	—
2		—	+	—	—	—
5	6	+	+	—	—	—
1		+	—	+	—	—
4		+	+	+	—	—
1		+	—	—	+	—
1		+	+	—	+	—
Total 62						

+ indicates excitation, — lack of excitation

in 8 % of the IC sample Among the EC recorded cells only one had convergence from 2 nerves The excitatory group I convergence of the total IC-EC sample is shown in Table II In cells receiving group I excitation from 2 or 3 nerves, the excitation from one of the nerves usually arrived at least 0 5 ms earlier than from the other(s) Occasionally, however, it arrived at the same short latency from two nerves as found in one cell excited by DR and Bic (6 ms)

Fore- and hind limb group I excitatory convergence occurred in 2 out of 62 cells Records from one such cell are illustrated in Fig 6 A, a—h showing the EC responses evoked by stimulation of the G S nerve at increasing strength At 1 3 T a double peaked EPSP is apparent which at 1 4 T reaches firing level The records of Fig 6 B show a wide excitatory convergence on this cell from forelimb group I- and SR afferents and from hind limb group I-, II- cutaneous and high threshold joint afferents

b) *Group II afferents* The excitatory convergence upon group I excited cells from forelimb group II afferents was rather prominent The group II effects were evident from increases in amplitudes of the EPSPs when the nerve was stimulated at group II strength or from additional later components not present on group I stimulation In the EC recordings 1—3 late spikes appeared on group II stimulation Group II afferents activated 12 of the 16 EC studied cells (75 %) and evoked EPSPs in 32 out of 46 (70 %) IC studied cells analyzed with special regard to group II contribution In 19 cells (41 %) the group II-EPSPs reached triggering level The excitation was mediated both by the same nerve supplying the group I excitation and/or from other forelimb nerves Records e—h of Fig 4 show IC potentials of a cell activated by group II afferents of the Tri nerve after a latency of 10 ms The same cell was also excited by volleys in group I DR afferents after a latency of 8 ms (record of this group I-EPSP not shown in Fig 4) In 23 (50 %) of the IC studied cells and in 6 (37 %) of the EC sample the group II effects were evoked from the same nerve supplying the group I excitation

As the group II-EPSPs in many cells were evoked by more than one forelimb

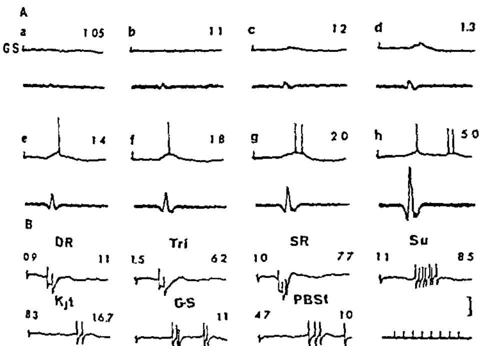


Fig 6 Extracellular records from a cell located in the medial part of the group I locus deep in ASSS and showing group I co-excitation from fore (DR and Tri) and hind limb (G S) afferents A upper records unitary responses evoked by G S lower records afferent volleys B records showing convergence of excitation on the same cell shown in A from fore- and hind limb afferents Voltage bar cortical potentials 1 mV Timer cortical potentials 5 ms

nerve latencies of 62 group II EPSPs were measured and found to range between 6.0 and 26.5 ms mean 13.1 ms (*cf* Table I) One third of them had latencies between 6.0 and 10.0 ms mean 8.4 ms One group II EPSP with a latency of 6 ms was evoked by Tri at high intensity in a cell also excited by group I DR afferents at the same latency In the EC sample the latencies of the first group II evoked spikes ranged between 9.5 and 25.0 ms mean 16.2 ms ($n = 17$) except in one cell with a latency of 6.5 ms The summit time of 30 group II EPSPs not reaching firing level was on the average 7.1 ms SD 3.1 (range 2.0 to 15.5 ms thus exceeding the corresponding mean of the total group I EPSP material with 1.3 ms The mean latency of the group II EPSPs exceeded that of the group I EPSPs with about 5 ms and the mean of the first group II evoked spikes that of the corresponding group I mean with about 6 ms The durations of the group II EPSPs exceeded in many cells 30 ms and the highest recorded amplitude was 7 mV The excitation from hind limb group II afferents was weak and rare in the group I excited cells The shortest observed latency of such EPSPs was 14 ms

The prominent contribution of excitation from group II forelimb afferents on group I activated cells—at times suggestive of monosynaptic linkage with group II thalamic relay neurones—suggests that the information from secondary endings in

muscles is functionally significant at the cortical level. It is, however, not excluded that the observed group II effects were evoked from afferents emerging from other receptors than secondary spindle endings.

c) *Skin afferents.* The IC records showed that every group I excited cell also received excitation from SR afferents (*cf.* Fig. 2 B, h and Fig. 4 b). This excitation was always evoked at stimulation strengths below 1.3 T. The SR EPSPs were similar in shape to those evoked by group I afferents, *i.e.* either simple or complex with up to six components observed. About 70 % of the SR-EPSPs reached firing level, the cells often discharging 2 spikes.

14 neurones were studied during gradual increase of stimulation of the cutaneous SR nerve. In 8 of them the EPSP was complex. The second component in 6 cells was evoked at 1.4 T or less and in the remaining 2 at 1.7 T. The amplitudes of 15 SR-EPSPs measured at 2 T ranged between 0.3 and 10 mV. In 34 SR EPSPs the summit times ranged between 1.0 and 9.0 ms, mean 3.7 ms, and in one additional cell it was less than 1 ms. The latencies of the simple SR-EPSPs and of the first component of the complex ones ranged between 5.2 and 16.0 ms, mean 8.4 ms (*cf.* Table I).

Of the 16 group I cells studied with EC recording 10 discharged 1—4 spikes on SR stimulation (*cf.* Fig. 2 A, h). More than half of them fired at 1.5 T or less and in some the thresholds were less than 2 T. The latencies of the first spikes ranged between 7.5 and 19.5 ms, mean 11.6 ms (*cf.* Table I).

As many of the SR-EPSPs had latencies of 7 ms or less (*cf.* Fig. 2 B, h and Fig. 4 b)—suggesting monosynaptic linkage with thalamocortical fibres—it was considered of interest to compare the latencies of the SR EPSPs with those of the group I EPSPs of the same cells and also to compare the latencies of the two types of EPSPs in neurones assumed to be activated monosynaptically by thalamic group I cells. There were 13 cells with latencies suggesting monosynaptic group I and di- or polysynaptic cutaneous linkage from thalamic relay cells. These cells are referred to as *primary cortical group I cells.* In 9 other cells both the group I and the SR latencies suggested monosynaptic excitation by thalamocortical axons. These cells are called *primary cortical mixed cells.* A third group of 10 cells in which the latencies suggested monosynaptic activation by cutaneous and di- or polysynaptic excitation by group I thalamocortical axons are labelled *primary cortical cutaneous cells.*

In the *primary cortical group I cells* the latencies of the SR EPSPs were between 0.5 and 5.6 ms longer (mean 3.1 ms) than those of the group I EPSPs. The latencies of the group I and SR EPSPs in the *primary cortical mixed cells* were equal or differed by less than 0.5 ms. In the *primary cortical cutaneous cells* the latencies of the SR EPSPs were between 0.5 and 4.0 ms shorter (mean 1.5 ms) than those of the group I-EPSPs. These observations suggest that group I afferents are connected to cortical neurones of different types. The *primary cortical group I cells* and the *primary cortical mixed cells* could represent proper receivers and relay cells of group I information from the periphery. They may belong to two different paths, one with

a "direct", the other with a delayed linkage to cutaneous afferents The *primary cortical cutaneous cells* may on the other hand be thought of as links in a cutaneous path which receives a delayed excitatory convergence from group I afferents

All three types of short latency *primary cortical cells* were characterized by group I input chiefly from one muscle nerve They were not excited by hind limb group I afferents They did however receive excitation from other fore and hind limb afferents The *primary cortical cutaneous cells* and the *primary cortical mixed cells* received more convergence from fore- and hind limb group II cutaneous and high threshold joint afferents than did the *primary cortical group I cells* These afferents have been referred to as flexion reflex afferents FRA by Eccles and Lundberg (1959) and by Holmqvist Lundberg and Oscarsson (1960) Forelimb group II excitation was provided both by the same nerve supplying the group I effect as well as from other nerve(s) The *primary cortical cutaneous* and *mixed cells* further resembled long latency group I cells in which excitation from FRA also was common. The *primary cortical group I cells* accordingly had a more simple convergence pattern than the two other *primary* groups

Hind limb skin afferents evoked responses in 27 of the 50 IC studied group I cells In 11 of them the Su EPSPs reached firing level discharging 1—2 spikes In 18 cases low threshold Su afferents excited the cells and in 9 other cases high threshold Su afferents The latencies of the Su EPSPs ranged between 11 and 46 ms mean 23 4 ms The highest observed amplitude of a Su EPSP was 2 mV

d) *High threshold joint afferents* Low threshold joint afferents did not influence group I cells of ASSS Excitation from high threshold Ejt afferents was observed in 20 of the 50 IC studied group I excited cells 10 of them discharged action potentials the other 10 showed EPSPs The shortest latency of an Ejt EPSP was 10 5 ms the highest amplitude was 8 mV In the EC sample only 2 out of 16 cells discharged spikes on Ejt stimulation The latencies of low amplitude Kjt EPSPs in 15 cells were 13 ms or longer and one of these cells discharged spikes on high intensity Kjt stimulation

e) *Auditory and visual input* Only subliminal excitation was evoked by these afferents in the group I cells Click stimulation evoked EPSPs in 3 cells with a shortest latency of 8 5 ms Visual afferents evoked long latency EPSPs in 5 cells

f) *Extra versus intracellular convergence patterns* The responses obtained with EC and IC recordings from the same cell were compared in 14 neurones The left columns of Fig 7 show the three types of EC classified neurones and their convergence patterns 4 cells (□) were EC identified as group I cells 6 cells (□) discharged spikes on SR and group II stimulation and 4 cells (□) only on SR stimulation The right columns of Fig 7 illustrate the detailed IC convergence patterns Thus all 14 cells received group I excitation The 4 group I DR cells showed on IC recording the same group I excitation originally found on EC recording except one cell in which an additional group I EPSP was evoked by B c A considerable increase in excitatory convergence from forelimb group II skin and high threshold joint afferents was however disclosed on IC recording from the 14 cells

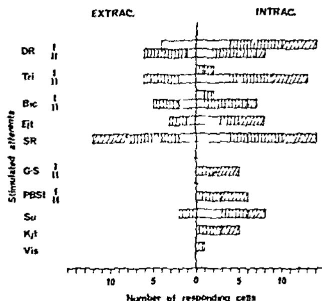


Fig 7 Diagram showing the difference in excitatory convergence observed with extra and intracellular recordings in 14 cells. The stimulated afferent nerves are indicated to the left I and II refer to group I and group II muscle afferents The length of each column indicates the number of cells responding to each of the given nerves Because of convergence, each cell may appear in more than one of the different columns Classification of cell types based on EC observations ☐ group I excited cells, ☐ cells activated by forelimb group II and SR afferents, ☐ cells excited by SR afferents

Similarly, excitatory convergence from hind limb FRA, almost absent on EC recording was revealed by the IC analysis

5) Inhibition of group I cells

a) Group I afferents 9 neurones were inhibited by group I afferents In 5 cells the IPSPs were uniform initial hyperpolarizations These cells did not receive group I excitation In the 4 other cells the postsynaptic potential was composed of two successive hyperpolarizations separated by a depolarization The last mentioned type represents neurones with mixed group I inhibition-excitation Records from a cell with a mixed response are shown in Fig 8 a—f The IPSP-EPSP complex was evoked by DR group I afferents The cell was also inhibited by DR group II afferents (*cf* Fig 8 e—f) as well as by group II afferents from the other forelimb nerves (records not shown in Fig 8) and by SR afferents, Fig 8 g—h The group II-IPSP thus counterbalanced the group I-EPSP in this cell Record i of Fig 8 shows an IPSP with a threshold of 1 0 T evoked by DR in another neurone, the IPSP potential being photographed during high intensity stimulation

Most of the group I-IPSPs were identified by visual inspection of the CRO, since cells responding with such IPSPs lasted only for a short while They were more difficult to maintain than the cortical cells inhibited by cutaneous afferents The cells with IPSPs evoked by skin afferents lasted considerably longer and were more stable This may indicate that the inhibited cortical group I cells are smaller than the cells inhibited by skin afferents The thresholds of the group I-IPSPs ranged between 1 0 and 1 6 T mean 1 2 T (an IPSP evoked at 1 6 T was included because of its short latency, 6 ms) The latency range of the group I IPSPs was between 6 0 and

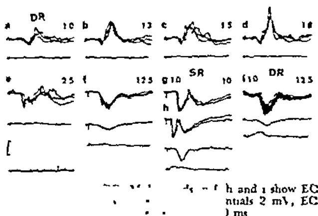


Fig 8 IPSPs in two cortical neurones of the group I locus of ASSS Upper records a—h obtained from a cell located 1740 μ deep and the upper record i from another cell 1600 μ deep in the cortex Resting potential of cell a—h 30 mV, that of cell i 38 mV a—d show IPSP—EPSP evoked by group I DR volleys and e—f an additional late IPSP evoked by group II DR afferents g—h composite IPSP evoked by SR threshold of second component not measured i a group ... h and i show EC ... ntials 2 mV, EC ...) ms

17 0 ms mean 9 5 ms (n = 10, *cf* Table I) The shortest IPSP lasted about 15 ms, many were longer than 30 ms The peak amplitudes were reached after about 5 ms and the decays were slower The highest amplitude recorded at supramaximal group I stimulation strength was 5 mV

Inhibitory group I convergence from two muscle nerves was found in 1 out of 9 cells IPSPs evoked by hind limb group I afferents were not observed

b) Group II afferents Cells excited by group I afferents were generally not inhibited by group II afferents Group II afferents did however, evoke IPSPs in all 9 cells inhibited by group I afferents (*cf* Fig 8) In 6 cells the IPSPs were evoked by two nerves in 3 by 3 nerves For technical reasons a possible group II inhibition from a nerve supplying group I IPSPs was not studied in detail 3 cells received mixed group II IPSP EPSPs The group II IPSPs had latencies between 8 5 and 22 0 ms mean 13 9 ms (n = 15) indicating that all group II IPSPs were generated via paths with a larger number of synapses The highest recorded amplitude of the group II-IPSPs was 2 3 mV and their durations were in general longer than those of the group I-IPSPs In 2 cells did group II hind limb afferents evoke long latency IPSPs

It is concluded that the group II effects excitation as well as inhibition in general seem to enhance the group I effects evoked in the cells of the group I locus of ASSS

c) Cutaneous afferents No IPSPs evoked by SR or Su afferents were observed in group I excited cells It can however not be excluded that IPSPs evoked by skin afferents could be concealed in complex EPSPs SR afferents did on the other hand evoke IPSPs in all 9 cells inhibited by group I afferents In 4 cells the SR IPSPs were uniform in the other 5 composite or mixed with EPSPs (*cf* Fig 8 g—h) The thresholds of the SR IPSPs were about 1 0 T and their latencies ranged between 6 and 13 ms mean 8 2 ms (*cf* Table I) In 4 cells the latencies ranged between 6 and 7 ms and in one cell the latencies of the SR- and the group I IPSPs were equal 6 5 ms In 3 other cells the latencies of the SR IPSPs were 1 to 4 ms shorter than those of the group I IPSPs The durations of the uniform SR IPSPs varied between 9 and

28 ms, in the composite IPSPs the duration of the first component varied between 8 and 15 ms and that of the second one between 30 and 50 ms (*cf* Fig 8 g—h) Mixed SR IPSP-EPSPs were observed in 2 cells, in one of them the EPSP preceded the IPSP by 5 ms

Hind limb skin afferents converged with IPSPs on 5 cells at latencies between 14 and 23 ms They were all rather uniform and of moderate amplitude In 2 other cells inhibited by group I- and SR afferents, Su evoked EPSPs at latencies of 10 and 23 ms

It is concluded that the effects from SR afferents on the group I cells are mainly enhancing the group I excitation as well as the inhibition This conclusion is, however, partly based on negative evidence since IPSPs evoked by skin afferents could be concealed in the composite SR-EPSPs of the group I cells

d) *High threshold joint afferents* A long latency Ejt IPSP was observed in one of the 9 cells inhibited by group I afferents Another group I inhibited cell was excited by Ejt at a latency of 12 ms Kjt evoked IPSPs were not seen, nor did auditory and visual afferents evoke IPSPs

6) *Cells not influenced by group I afferents*

63 cells (47 %) of the group I locus were uninfluenced by group I afferents 51 were excited ... EC ... the ... up I ... ms ... ion ... the

ncies were 10 ms or longer One cell was excited by visual input at a latency of 93 ms

Excitation from forelimb group II afferents Weak excitation from group II afferents was seen in 4 cells Only one of them had convergence from two nerves The latencies were 16 ms or longer

Co excitation from forelimb group II and skin afferents 26 cells were excited by forelimb group II and SR afferents IC recordings were obtained from 2 cells SR afferents evoked more regular discharges than group II afferents and was of low threshold in all except one cell (2T) The group II excitation was mediated in 13 cells by one nerve in 12 by two and in one cell by three nerves The latencies of the first SR evoked spikes ranged between 5 and 15 ms mean 10 7 ms (4 cells had latencies between 5 and 6 5 ms) The latencies of the first spikes evoked by group II afferents in 40 instances ranged between 7 and 30 ms mean 16 2 ms (the group II potentials with latencies between 7 and 9 ms were evoked in the same cells which received short latency SR excitation) Weak long latency excitation from high threshold Ejt afferents was seen in a few cells

The short latency group II and SR potentials suggest that some cells were monosynaptically linked to thalamocortical afferents These cells may therefore represent another group of *primary cortical mixed cells cf* pp 12—13

Excitatory convergence from forelimb and other afferents Excitation from fore- and hind limb afferents was seen in 9 cells All except one were excited by low threshold SR afferents at latencies between 7 and 21 ms Group II forelimb excitation was mediated in 5 cells by one nerve in 3 by two and in one cell by three nerves The latencies of the first group II spikes ranged between 8 5 and 26 ms 2 cells were excited by high threshold Ejt afferents after long latency The excitation from hind limb afferents was weak and mainly evoked at shortest latency from Su afferents (14 ms) In a small number of cells excitation from auditory and ... convergence ...) and 900 μ ... 16 ms) and ... SR afferents

by low ... (latency 8 5 ms) but excited by forelimb group II afferents from three nerves at latencies between 8 5 and 19 ms Ejt activated that cell at long latency

Discussion

Ascending paths The present observations indicate that the path from forelimb group I muscle afferents to the cerebral cortex of ASSS is a fast one, characterized by short latency and efficient synaptic linkages A large number of cells in this group I locus received group I excitation after a latency which indicated monosynaptic connexion with thalamocortical axons These cortical cells are therefore fourth order neurones in the ascending group I projection as was previously suggested for the group I cells of Pcd by Oscarsson *et al* (1966)

The group I cells of ASSS with latencies between 5 and 7 ms are assumed to be monosynaptically activated by thalamic group I relay cells This assumption is based on comparisons with the short latency excitation of group I cells of Pcd and with the latencies of the excitation of thalamocortical group I relay cells (Rosen 1969c) In Pcd the delay between the small positive surface wave signalling the arrival of the thalamocortical volley and the cortical postsynaptic potential is easily determined In the recordings in ASSS the incoming volley is often not distinct enough to allow measurements on synaptic delay Rosen (1969c) showed that the latencies of the first group I evoked spikes in thalamic relay cells ranged between 2.7 and 4.9 ms when measured from the arrival of the incoming volley at C_5 Allowance for additional minimal conduction time in corticopetal axons of 0.5 ms plus a synaptic delay of 0.5 ms, and an additional 1.5 ms for primary afferent conduction to C_5 would include group I activated cells in the ASSS locus with latencies between 5.2 and 7.4 ms in the sample considered monosynaptically activated by thalamic group I discharges

According to the observation of Andersson Landgren and Wolsk (1966) thalamic group I relay cells send bifurcating axons to ASSS and Pcd Rosen (1969c) demonstrated however, that the majority of the thalamocortical group I relay cells projected only to one of these areas A bifurcating group I thalamocortical axon would convey identical information to the neurones of the two cortical areas It is therefore of interest to find out if cortical group I cells with identical afferent excitatory convergence are found in ASSS and Pcd A comparison between the present material and that of Oscarsson *et al* (1966) shows that such cortical cells do exist They form approximately 20 % of this ASSS sample and are characterized by monosynaptic group I and at least disynaptic cutaneous linkage with thalamocortical axons

Certain apparent differences between the ASSS and Pcd samples were observed Thus all group I excited cells of ASSS received a cutaneous input This was not the case with the Pcd group I cells Furthermore 13 % of the monosynaptically group I activated cells of ASSS and 18 % of the di or polysynaptically group I excited cells were also provided with a monosynaptic thalamocortical cutaneous input In Pcd only di or polysynaptic thalamocortical cutaneous excitation was seen in the group I cells (Oscarsson *et al* 1966 Swett and Bourassa 1967

A difference in excitatory group I convergence was also found in the two cortical areas as Table III illustrates The majority of the ASSS cells received a group I input restricted to one muscle nerve In this respect they were more like the thalamic

TABLE III Comparison between patterns of excitatory convergence from forelimb group I afferents in different group I loci of the ascending group I projections The comparison is made only between the excitation evoked by DR, Tri and Bic nerves observed in the main cuneate nucleus (Rosén 1969b Table I), the thalamus (Rosén 1969c Table I), the Pcd (Oscarsson et al 1966, Table II) and in the group I locus of ASSS of the present material (Table II)

Number of cells		(%)	main cuneate nucleus Stimulated afferent DR	Tri	Bic	Convergence
0			+	+	+	3
0	10	(22)	+	+	—	2
10			+	—	+	2
28			+	—	—	1
4	36	(78)	—	+	—	1
4			—	—	+	1
Total 46						

Number of cells		(%)	thalamus Stimulated afferent Dr	Tri	Bic	Convergence
0			+	+	+	3
4	6	(16)	+	+	—	2
2			+	—	+	2
28			+	—	—	1
2	32	(84)	—	+	—	1
2			—	—	+	1
Total 38						

Number of cells		(%)	Pcd DR	Tri	Bic	Convergence
2		(10)	+	+	+	3
3	8	(40)	+	+	—	2
5			+	—	+	2
3			+	—	—	1
6	10	(50)	—	+	—	1
1			—	—	+	1
Total 20						

Number of cells		(%)	ASSS locus DR	Tri	Bic	Convergence
4		(6 5)	+	+	+	3
6	7	(11 5)	+	+	—	2
1			+	—	+	2
49			+	—	—	1
2	51	(82)	—	+	—	1
0			—	—	+	1
Total 62						

group I relay cells (Rosen 1969 c) than the group I cells of Pcd in which convergence from two or three nerves was common

A thalamocortical inflow via bifurcating group I axons cannot explain the observed differences between ASSS and Pcd group I cells Additional input channels must be assumed Fig 9 shows some hypothetical afferent connexions to the short latency group I cells of ASSS Alternative A fits the observation that group I cells in ASSS and Pcd are monosynaptically excited by thalamocortical group I axons and at least disynaptically activated by corresponding low threshold cutaneous fibres The convergence between group I and cutaneous excitation may take place already in the main cuneate nucleus (Rosen 1969 b) Alternatives B C and D would be compatible with the finding that ASSS cells are excited monosynaptically by cutaneous as well as by group I thalamocortical axons Rosen (1969 c) observed that 60 % of the thalamic group I relay cells did not receive cutaneous excitation from cuneothalamic fibres Those cells which were discharged by cutaneous afferents responded with a longer latency suggesting an extra synapse in the cutaneous path This was also true for the cuneothalamic group I relay cells For these reasons, alternatives B and C should be given preference over D As only a minority of the

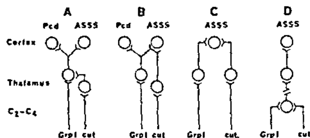


Fig 9 Diagrams of hypothetical excitatory coupling A—D for ascending group I projection at the levels of cervical spinal cord (C_2—C_4 Rosen 1969 a, b), thalamus (Rosén 1969 c), cerebral cortex Pcd (Oscarsson *et al* 1966) and cerebral cortex, group I locus in ASSS (present study) Grp I = group I, and cut. = low threshold cutaneous forelumb paths

thalamic group I cells projected to the ASSS undivided axons (Rosén 1969 c) alternative B should finally be accepted as the most likely arrangement It was mentioned above that the monosynaptic excitatory convergence between group I- and cutaneous afferents did not occur in the main cuneate nucleus Rosen (1969 a b) described, however, such convergence on group I cells located in the dorsal horn at C_2—C_4 level Ascending projection from those cells has so far not been demonstrated

Excitation The low group I thresholds of neurones in ASSS locus suggest that primary spindle afferents influenced the cells It is, however, possible that also Golgi tendon organ afferents contributed to the evoked cortical responses Only adequate activation of these afferents will provide evidence for or against their influence on group I cells of ASSS

The differences in IC excitatory pattern entitle cells of ASSS monosynaptically linked to thalamocortical fibres to be grouped into three categories The *primary cortical group I cells* would constitute one group They were activated di- or polysynaptically by cutaneous thalamocortical axons Among them group II co excitation was rare and of long latency The simplest and also most common type of cell observed was provided with excitation from group I afferents of one muscle nerve and from low threshold skin afferents These cells therefore integrate spatially restricted information from muscle spindle primary endings with information from skin receptors of the paw The cells monosynaptically activated by group I as well as by skin thalamocortical fibres would be *primary cortical mixed cells* Excitation from group II afferents was more common among these cells than in the previous group Occasionally the latency of group II responses suggested monosynaptic linkage with thalamic cells The convergence patterns onto the majority of the cells were simple, *i e* they were excited by group I afferents of one nerve and by skin afferents

In the above mentioned two groups there were cells in which a higher degree of integration took place Such cells received monosynaptic thalamocortical excitation from two or three nerves or in other cases monosynaptic group I excitation from one nerve and polysynaptic from the other nerves

The third type of cortical group I excited cells consisted of those monosynaptically connected to cutaneous thalamocortical axons but not to group I axons These neurones were therefore called *primary cortical cutaneous cells* The latencies of the group I excitation of these cells suggested di- or polysynaptic linkage from th

cells Most cells received their group I information from one nerve These cells were also to a considerable degree excited by group II afferents both from the same nerve which supplied the group I excitation as well as from other nerve(s)

The functional implication for the existence of two types of cutaneous excitation of *primary cortical cells* one being mono the other at least disynaptically linked to thalamocortical afferents is not understood In both instances however the cutaneous afferents may emerge from Type I endings in hairy skin (Iggo and Muir 1969) Brown and Hayden (1971) reported that the afferents of sural Type I endings in the rabbit were electrically excitable at or below 1 3 T The IC thresholds of the SR evoked synaptic potentials in many of the group I cells of this study had similar low values

Inhibition Only a few group I inhibited cells were studied Their shortest latencies —at least 1 ms longer than those of the corresponding EPSPs—indicated though in conformity with the interpretation given by Oscarsson *et al* (1966) that these IPSPs were evoked by fourth order cortical group I excited cells Skin afferents evoked IPSPs which in some cells had the same short latency suggesting disynaptic linkage with thalamocortical axons Only long latency group II IPSPs were seen and they were often evoked from nerves not supplying the group I inhibition Inhibitory group I convergence from two nerves was observed in 1 out of 9 cells This contrasts with the findings of Oscarsson *et al* (1966) of extensive group I inhibitory convergence found in Pcd cells

Location The primary cortical cells of ASSS were considered monosynaptically linked to thalamic relay cells It is relevant to find out if they are located in those cortical layers where the thalamocortical fibres mainly end Specific thalamic afferents have been shown to terminate chiefly in layers III—IV (Lorente de No 1949 Nauta 1954 Sholl 1955) The *primary cortical group I cells* were found at depths between 350 and 2000 μ presumably in layers II—VI The *primary cortical mixed cells* were encountered at depths between 1000 and 1700 μ i e from layer IV down The *primary cortical cutaneous cells* were located at depths between 800 and 2000 μ i e in layers III VI

These results indicate that the cells of ASSS locus directly linked to thalamocortical fibres were not confined only to layers III—IV shown to receive specific thalamic afferents Such afferents have previously been considered to terminate mainly on pyramidal cells Recently however Marty and Fuentes (1968) showed that the apical dendrites of the pyramidal cells as well as the dendrites of the stellate cells of layers III—VI in the auditory cortex showed degenerative changes after interruption of thalamocortical afferents In the somatic sensory cortex Jones and Powell (1970) found in addition to the main degeneration in layer IV some degenerative changes in the molecular layer Garey (1970) interpreted the degeneration predominantly in layer IV of area 17 as suggestive of synapses on the spines of stellate cells The wide depth distribution of cortical cells of the ASSS locus monosynaptically linked to thalamic relay cells would thus have anatomical correlates in pyramidal and stellate cells

The cells inhibited by group I afferents were located approximately in layers IV—VI If the IPSPs were recorded from cells with intracortical contacts as the Pcd findings of Oscarsson *et al* (1966) suggest these cells may correspond to the pyramidal cells with axon collaterals (P_4) and cells with intracortical distributions (S_1, S_3) of Sholl (1955) The inhibited neurones may also be represented among cortical "short local neurons' of Szentagothai (1965) remaining in chronically isolated cortical slabs

Function The present knowledge of the afferent connexions to group I cells of ASSS is not sufficient to explain in which cortical mechanisms these neurones participate Abrahams (1969) reported that electrical stimulation of Pcd and ASSS regions potentiated monosynaptic lumbo-sacral reflexes much in the same manner as did stimulation of neck muscle and forelimb muscle and skin nerves The ASSS region was unique in that after its ablation virtually no potentiation of reflexes could anymore be elicited from stimulation of the above mentioned peripheral nerves Group I cells of ASSS may accordingly take part in cortical mechanisms integrating postural reflexes Such a mechanism was in fact suggested by Abrahams for the region of ASSS The ASSS region also receives afferent inflow from neck muscles (Landgren and Silfvenius 1968) and vestibular afferents (Walzl and Mountcastle, 1949, Mickle and Ades 1954 Andersson and Gernandt 1954 Landgren *et al* 1967 b) Whether single group I cells of ASSS locus are linked to afferents from neck muscles and to vestibular afferents has not been investigated Convergence on the focal potential level in the group I locus of ASSS between group I and vestibular afferents was however, observed at the caudal border of the group I locus (Landgren *et al* 1967 b)

I thank Professor Sven Landgren for his advice and criticism Miss Ewa Lignell gave technical assistance during the experiments performed at the Department of Physiology, University of Goteborg

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DEMONSTRATIONS

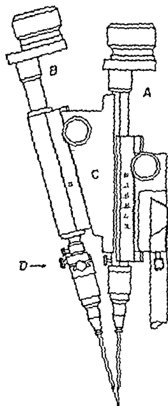
D 1

Single Unit Discharges in Brain Slices Maintained *in vitro*

By P ANDERSEN, B BLAND, K SKREDE, O SVEEN and R WESTGAARD, *Institute of Neurophysiology, University of Oslo, Norway*

Yamamoto and McIlwain (1966) reported that slices from the prepiriform cortex could be maintained *in vitro*, and still show electrically evoked potentials comparable with those of the intact animal With the many advantages offered by an *in vitro* preparation of brain tissue in mind, we wanted to know whether single unit recording was possible, and, if so, what properties do single neurones in a brain slice show, compared to the same type of cells in an intact animal? We have investigated slices from different areas of the brain, concentrating on the transverse hippocampal slice (Skrede and Westgaard 1971) The slices (300 to 500 μm thick) were kept in a constant flow incubation chamber (Richards and Sercombe 1970) in an oxygenated fluid having the following composition in mM NaCl 134, KCl 5, KH_2PO_4 1 24, $MgSO_4$ 1 3, $CaCl_2$ 0 75, $NaHCO_3$ 16 and glucose 10 Activity of a single cell could be recorded for at least 2—3 h The stability of the recording condition allows long term examination of the behaviour of the units, *e g* studies of the variation of the discarge pattern of a spontaneously firing unit, or the firing pattern following electrical stimulation of an appropriate pathway Two types of pulses were fed to a computer for each sweep of the oscilloscope One was locked to a point just after the start of the sweep, usually near the stimulus artefact, and used for timing purposes This facility was used for analysis of oscilloscope sweeps, but was not necessary for spontaneous activity The other pulses were triggered by the unit discharges after appropriate amplification and fed to the computer on a separate channel To facilitate the necessary discrimination the pulses were displayed on an oscilloscope just under the unit discharges

The results of the unit analysis were presented in the form of interval histograms auto- or cross correlation plots, series correlation plots or post-stimulus histograms The plots were either written on a teletype or in graphic form on a storage oscilloscope For the long-term studies of unit latencies a cumulative plot of individual spike latencies was particularly instructive Spontaneous unitary discharges were regularly found in the slice preparations This holds true both for the pyramidal and the granule cells of the hippocampal formation Spontaneous unit discharges were recorded in most thalamic slices as well



Schematic drawing of manipulator system A and B are two micromanipulators joined at 15° angle B is constructed according to Eide and Kallstrom (1968) A is a new version of this construction with access to one side of the mo ing slide to which is fixed a wedge shaped support C wl ich in turn holds B The whole system is attached to a rig d stand (to the right) with possibilities for manual adjustments of angle and position Each manipulator carries an input amplifier holding a microelectrode B also carries a com pound slide arrangement D for small sideways adjustments of the electrode position

D 4

Double Micromanipulator for Independent Impalements of One Neurone with Two Electrodes

By I Engberg Y Kallstrom and K C Marshall Department of Physiology University of Goteborg Sweden

In analyses of neurone membrane functions double intracellular microelectrodes are used for experimental alterations of membrane potential one electrode for current injection one for recording In CNS investigations the two electrodes are usually joined with the tips close together giving them a large common resistance The voltage drop over this coupling resistance created by current injection adds to the recorded membrane potential shift Compensation for this voltage on the assumption that the coupling resistance is constant in the intracellular and extra cellular media is in our experience not reliable The coupling resistance and thereby the potential measurement errors should be reduced by the ... dent intracellular electrodes

We will demonstrate a manipulator system which faci ... of 2 microelectrodes independently into the same neurone ... of 2 remotely controlled electrical manipulators (A and B in th ... g

ere microelectrode The manipulators are joined so that when A is operating both electrodes move without any change in their relative positions Before inserting the electrodes into the tissue their tips are aligned under microscopic control by operating manipulator B and a small compound slide arrangement (D) After the alignment the one electrode is withdrawn by manipulator B to permit tracking for neurones with the other Upon impalement of a neurone, manipulator B is advanced towards the alignment position until its electrode penetrates into the same cell We have proved the usefulness of this technique in recording from superficially located spinal motoneurones with a dorsolateral approach to the ventral horn It may be applicable to other situations where precise relative positioning (extra cellular or intracellular) of two separate electrodes is desirable

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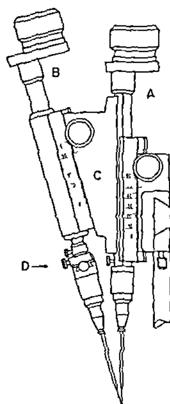
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D 5

A Cutaneous Projection to the Pyriform Cortex in the Cat

By S A Andersson and M Santini *Department of Physiology University of Göteborg, Sweden*

Except for olfaction, it is not known if sensory modalities that are represented in the amygdala (Machne and Segundo 1956) also project to the pyriform cortex We have recently found that cutaneous afferents from the superficial radial nerve (SR) have this projection responses to contralateral stimulation being larger than those to ipsilateral stimulation These experiments were performed in chloralose and nembutal anaesthetized cats with exposure of the entire pyriform cortex Similar results were obtained with both types of anaesthesia provided that the nembutal dose was moderate The pyriform cortical response to SR stimulation exhibited its largest amplitude in a transverse band of the intermediate two quarters of the cortex, 2—3 mm behind the caudal border of the olfactory tubercle and the orbital cortex The typical response was negative positive (positive larger) and the latency from stimulus onset to the negativity developing above the baseline was 20—25 ms These pyriform responses developed at a stimulus strength slightly above that necessary to evoke a concomitantly recorded primary response from the somatosensory (S_I) cortex, suggesting that the response was attributable to the larger SR fibres The responses were also frequency sensitive being maximal at stimulus frequencies lower than 1/5 s and absent at 5/s In contrast to low threshold stimulation of the SR, supramaximal stimulation of the contralateral and ipsilateral deep radial nerves was required to evoke small potentials in the pyriform cortex Large evoked responses to olfactory stimulation were also observed at sites exhibiting the SR responses This overlap suggests potential interaction between olfactory and cutaneous inputs



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D 4

Double Micromanipulator for Independent Impalements of One Neurone with Two Electrodes

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In analyses of neurone membrane functions double intracellular microelectrodes are used for experimental alterations of membrane potential one electrode for current injection, one for recording In CNS investigations the two electrodes are usually joined with the tips close together giving them a large common resistance The voltage drop over this coupling resistance created by current injection adds to the recorded membrane potential shift Compensation for this voltage on the assumption that the coupling resistance is constant in the intracellular and extra cellular media is in our experience not reliable The coupling resistance and thereby the potential measurement errors should be reduced by the use of two independent intracellular electrodes

We will demonstrate a manipulator system which facilitates the insertion of 2 microelectrodes independently into the same neurone The system consists of 2 remotely controlled electrical manipulators (A and B in the figure) each carrying

Whether the vessels of SHR react with a more extensive structural change to a given pressure load cannot be settled by these studies alone, even a small difference in this respect may in the long run be hemodynamically quite important

Reference

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D 7

Cardiovascular Responses to Acute Mental "Stress" in Spontaneously Hypertensive Rats (SHR)

By B Folkow, M Hallbäck and L. Weiss *Department of Physiology, University of Goteborg Sweden*

Several findings point to central neurohormonal influences as being of importance for the development of hypertension in SHR (Okamoto 1969) Such functionally elicited pressure increases may also initiate the structural adaptation of the resistance vessels which seems to be crucial for establishing a truly hypertensive state both in SHR and in primary hypertension in man (see Folkow 1971)

The present study was designed to explore whether SHR were "hyperreactive", compared with normotensive control rats (NCR) with respect to the hypothalamic defence reaction as provoked under identical control situations by graded "stressful stimuli (sudden light sound vibration) 20 expts were performed on pairs of awake SHR—NCR placed in an especially designed double box while blood pressure and heart rate were recorded continuously via the tail arteries After an hour of adaptation the animals were exposed to identical stimuli where vibration proved particularly efficient for eliciting a substantial and reproducible defence reaction The characteristic heart rate increase forming part of this reaction and known to involve both an accentuated sympathetic discharge and a centrally supressed vagal tone was here preferentially utilized to judge the intensity of the defence reaction since the pressor response will be potentiated in SHR also because of the increased wall/lumen ratio of their resistance vessels The relative roles of sympathetic and vagal modulations of heart rate were judged by comparing responses before and after supramaximal i a administrations of a β blocker (Aptin® followed by atropine or *vice versa*

These drug effects strongly suggested an enhanced sympathetic discharge in SHR even in the control situation but also this situation no doubt involves some mental "stress , or enhanced alertness SHR tended to respond stronger than NCR to all stimuli used both in pressure and heart rate but the responses to vibration were easiest to analyse because of their better consistency and larger deviation from the baseline Also after complete adrenergic blockade of the heart, this type of sti-

mulus elicited a decidedly stronger tachycardia in SHR ($p < 0.001$) Atropine virtually abolished this response which clearly indicates a more pronounced central suppression of vagal tone in SHR in connection with a defence reaction besides the mentioned evidence of accentuated sympathetic discharge These findings therefore suggest a true hyperreactivity in SHR of those central autonomic structures that mediate emotional behaviour during alertness and mental stress

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D 8

The Effect of Prolonged Propranolol Treatment on Blood Pressure and Structural Design of the Resistance Vessels in Young Spontaneously Hypertensive Rats (SHR)

By B Folkow Y Lundgren and L Weiss *Dept of Physiology University of Goteborg Sweden*

Results by Okamoto *et al* (Okamoto 1969) suggest that neurohormonal excitatory influences of central origin are of importance for the development of hypertension in SHR The pattern may be related to the hypothalamically integrated defence reaction which may also be the case with that observed in early primary hypertension of man Thus the pressure rise is here associated with an increased cardiac output and muscle blood supply

Against such a background SHR were given propranolol in order to interfere primarily with the cardiac output About 100 mg/kg and day was given in the drinking water starting at the age of 8 weeks and lasting for about 8 months during which period blood pressure was intermittently checked by a tail plethysmographic method In acute experiments both pressure and heart rate were measured during nembutal aneasthesia via the cannulated tail artery While mean blood pressure was 184 ± 5 mm Hg in untreated matched SHR it was only 134 ± 9 mm Hg in the treated SHR as compared with 132 ± 3 mm Hg in untreated normotensive control rats (NCR) Resting heart rate was about 15 % lower in the treated SHR as compared with the untreated controls

In 10 paired experiments the hindquarters of one treated SHR and one untreated NCR were simultaneously perfused at constant flow with a plasma substitute starting from maximal dilatation Noradrenaline (NA) was then infused in a step wise fashion to give the entire dose response curve for both vascular beds The maximal pressor response reflecting the maximal contractile strength of the resistance vessels was induced by supramaximal slug injections of vasopressin and Ba Cl

While ordinary SHR compared with NCR exhibit a higher resistance even at maximal dilatation a steeper dose response curve and a raised maximal contractile

strength of the resistance vessels, but unchanged NA threshold (see Folkow 1971), the treated SHR showed more normalized hemodynamic characteristics in all these respects

The results thus indicate that a prolonged treatment of SHR with propranolol if started in the developing phase of hypertension, may largely prevent the hypertension and decrease the structural changes of the resistance vessels, which are otherwise the characteristic sign and probable background of an established hypertensive state

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D 9

5-Hydroxytryptamine and Intestinal Blood Flow

By B Biber, J Fara and O Lundgren, *Department of Physiology, University of Göteborg, Sweden*

Mesenteric blood flow has been shown to increase following instillation of fat or acid into the duodenum, involving the release and action of the duodenal hormones cholecystokinin (CCK) and secretin (Fara *et al* 1971) Intestinal vasodilatation has also been demonstrated in response to mechanical stimulation of the intestinal mucosa (Biber *et al* 1971), and in current experiments by electrical field stimulation across the wall of the intestine Thus it s likely that different trigger mechanisms are involved in the regulation of mesenteric blood flow

However, as we have also recently observed that close i.a injection of 5 hydroxytryptamine (5 HT) evokes an intestinal vasodilator response the present experiments were performed to investigate the possible role of 5 HT as a common intermediary vasoactive substance in the local control of mesenteric blood flow elicited by these various stimuli Recording venous outflow from the small intestine of the cat, we studied the effects of different receptor blocking agents on the blood flow responses to close i a infusion of CCK and secretin mechanical stimulation of the intestinal mucosa electrical field stimulation and to i a injection of 5 HT The intestinal vasodilatations evoked by these procedures were unaffected by α receptor blockade (phenoxybenzamine) β receptor blockade (propranolol) or cholinergic receptor blockade (atropine) However the 5 HT and α receptor antagonist dihydroergotamine given in doses sufficient to block the vascular effects of 5 HT but not completely the vasoconstrictor response to sympathetic nerve stimulation abolished the blood flow responses to CCK and secretin infusion and to mechanical and electrical stimulation of the intestine The 5 HT blockade during which the vascular bed was not maximally dilated as tested by the effects of papaverin was effective for 10—15 min By approximately 30 min the mesenteric vasodilata

in response to 5 HT and the other tested stimuli returned to control levels The experiments thus indicate that 5 HT, found in the intestinal wall (Vialli 1966), is essential in the physiological regulation of mesenteric blood flow

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D 10

Reflex Bradycardia Elicited from Left Ventricular Receptors during Acute Severe Hypoxia in Cats

By PETER THOREN *Department of Physiology, University of Göteborg Sweden*

Recent studies on cats (Thoren to be published) have shown that left ventricular receptors, firing in non medullated vagal afferents become activated during asphyxia This receptor excitation was in all probability, caused by an ischemic dilatation of the ventricle during asphyxia The present experiments were made to analyze whether these ventricular receptors may contribute to the reflex bradycardia seen during acute severe hypoxia in cats

Chloralose anesthetized animals were used

Blood pressure heart rate and left ventricular pressure were measured as well as changes in ventricular volume by means of a cardiometer Hypoxia was induced by ventilating the animal with a gas mixture containing 3 % oxygen The aortic nerves were cut bilaterally

With a latency of 1—2 min after the onset of hypoxia when arterial pO reached values between 14—34 mm Hg the ventricular diastolic pressure and volume increased and simultaneously a bradycardia developed This slowing of the heart was eliminated by cooling of the cervical vagi and reappeared upon rewarming indicating that nervous mechanisms rather than local myocardial factors are the main cause for the bradycardia during acute severe arterial hypoxia

To evaluate the role of the ventricular receptors for the reflex bradycardia during hypoxia, procain (0 3—0 5 %) was administered *into* the cardiometer In this way the majority of the *afferent* fibres from the ventricular receptors were blocked while the *efferent* vagal fibres to the sinus node remained intact as indicated by a strong bradycardia upon efferent vagal stimulation Such selective denervation of the ventricular receptors led to a marked reduction of the bradycardia response during hypoxia suggesting that ventricular receptors are considerably involved in this reflex response In some cases and particularly when the preparation started to deteriorate at the end of the experiment a marked bradycardia could be observed even after blockade of ventricular afferent fibres Thus also other mechanisms such as *e g* chemoreceptor reflexes or a direct influence of oxygen lack of central vagal neurons may therefore contribute to the bradycardia response during severe hypoxia

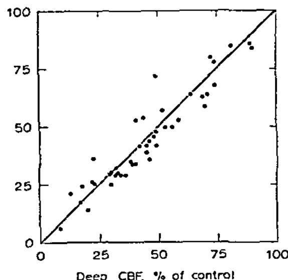


Fig 1 Effect of bleeding on superficial and deep cortical blood flow in per cent of control

D 11

Distribution of Renal Cortical Blood Flow during Hemorrhagic Hypotension in Dogs

By K. Aukland, A Kirkebo E Loyning and I Tyssebotn *Institute of Physiology, University of Bergen Norway*

During hemorrhagic hypotension, blood flow decreases more in kidney, muscle portal bed and skin than in organs such as brain and myocardium. Also within the kidney several studies suggest a redistribution of blood flow India ink injections and Xe washout recorded externally or by radioautography have indicated several patterns 1) Cortical ischemia with normal flow in medulla 2) Ischemia mainly in the outer cortex. 3) Patchy cortical ischemia (Carriere *et al* 1966 Trueta *et al* 1947, Truniger *et al* 1966) On the other hand local measurements of H_2-gas clearance and red cell transit time (Aukland and Wolgast 1968) showed proportional reduction of medullary blood flow and total renal blood flow but gave no information about the distribution within cortex The present experiments were designed to elucidate this point

Seven experiments were undertaken in 5 dogs in Nembutal anaesthesia Platinum electrodes were implanted in cortex 3 in outer cortex (2—4 mm deep) and 3 in inner cortex (5—8 mm deep) Hydrogen gas dissolved in saline was injected into the renal artery and local H_2-clearance measured polarographically (Aukland and Wolgast 1968) Total renal blood flow (RBF) was measured by electromagnetic flowmeter After a control period the dog was bled to an arterial pressure of 50 mm Hg, and maintained at this level for 1—2 h

RBF fell with decreasing pressure showing first a fall and then a rise in renal flow resistance Local flow averaged 3 5 and 3 6 ml/min g in outer and inner cortex respectively in control measurements During bleeding local flow fell by 60—90% proportionally in the two zones (Fig 1) and in proportion to RBF

Various electrodes in the same kidney showed considerable scatter (SD 0 6 ml/min g) in control measurements During bleeding SD fell approximately in proportion to flow, indicating a rather homogeneous ischemia

Conclusions Measurement of local hydrogen gas clearance indicates that hemorrhagic hypotension reduces blood flow proportionally in outer and inner renal cortex and shows no evidence for patchy ischemia

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D 12

A Difference between High and Low Drinking Rats in Effects of Ethanol on Ion Movements in Cerebral Tissue

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Ethanol inhibits especially the net movements of sodium ions during electrical stimulation and the active transport of potassium ions in the recovery period following stimulation in rat cerebral cortex slices (Nikander and Wallgren 1970) The purpose of the present experiments was to find out if the high-drinking AA rat strain differs from the low-drinking ANA strain (Eriksson 1971) in respect to the inhibition of the ion movements in the cerebral cortex induced by ethanol

The brain slices were prepared by means of the method of Wallgren and Kulonen (1960) The slices were incubated at 37 C in a buffered bicarbonate medium saturated with O Inulin was used as a marker of the extracellular space (Keesey Wallgren and McIlwain 1965 The slices were incubated for 30 min before the electrical stimulation to ensure equilibration of the ion distribution Carbon electrodes (Nikander, in press and alternating pulses of 14 V 28 mA) with a time-constant of 0 4 ms were used for the stimulation The ethanol was always added to give a concentration of 0.5 g/100 ml of the medium In examining the 10 minutes stimulation period ethanol was added just before the electrical stimulation In studying the recovery period the slices always were stimulated for 10 min after which they were allowed to recover for 10 min The alcohol was added just before the pulses were interrupted

During stimulation, the net movements of the sodium and potassium ions were inhibited in both the AA and ANA strains There was thus no difference between the two groups in this respect In the recovery phase no alcohol-induced inhibition of sodium ion transport could be seen in either of the rat strains The net gain of potassium ions was inhibited in brain slices from the ANA rats No such inhibition was to be seen in the AA rats

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D 13

Effect of Oxygen on Human Adipose Tissue Blood Flow (ATBF)

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Decompression of divers is conventionally based on tables calculated on the assumption that the body can be treated as composed of tissues that saturate and desaturate exponentially with different, but unchanging rate constants (Haldane and Priestly 1935) Improvements of current decompression procedure might be achieved, if changes in perfusion rates (and thus in saturation/desaturation rates) of various tissues could be taken into account Knowledge of blood flow variations in tissues with low rate constants ('slow' tissues) would be of particular interest since the slow tissues limit the rate of decompression after deep or prolonged dives Adipose tissue is the quantitatively most important slow tissue One factor that may influence the blood flow of a tissue during diving is the varying oxygen tension

In the present experiments the effect of oxygen breathing on abdominal subcutaneous ATBF was investigated in 12 normal men ATBF was measured by the ^{133}Xe washout method (Larsen, Lassen and Quaade 1966) An average value of 5 5 ml/100 g min was found during air breathing When oxygen was breathed at 1 atm this value was reduced by 20 % at an average limits 2—54 % This was the case whether air breathing was shifted to oxygen breathing or vice versa

The effect of oxygen on ATBF is similar to that described for a number of other tissues (Lambertsen 1966) Since the effect varied considerably between individuals it is concluded that standard diving tables cannot be modified so as to take it into account

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D 14

The Effect of Noradrenaline on the Splanchnic Elimination of Ethanol and Hepatic Excretory Function

By NIELS KRARUP, *Institute of Physiology, University of Århus, Denmark*

Sphincter activity in the hepatic sinusoids regulating the intrahepatic distribution of the blood flow has been described by several authors (see McCuskey 1966) The sphincter activity may depend on vasoactive agents including noradrenaline, which has been shown to increase the intrahepatic vascular resistance (Turk and Shoemaker 1962) An uneven distribution of the resistance changes elicited by noradrenaline may result in a decrease in the number of perfused sinusoids and a corresponding decrease in the functional capacity of the liver

In 15 chloralose anesthetized cats noradrenaline was infused continuously into a femoral vein at rates ranging from 0 5 to 5 0 μg/kg/min after a control period of 150 min The estimated hepatic blood flow (EHBF) was determined by means of Indocyanine Green (ICG), and mean arterial pressure was recorded from a femoral artery The functional capacity of the liver was determined by measuring the splanchnic elimination of ethanol at concentrations at which the elimination capacity is saturated as well as the flow and composition of bile Furthermore in 4 of the cats the splanchnic consumption of oxygen and output of glucose was determined

The splanchnic elimination rate of ethanol and the splanchnic consumption of oxygen was not changed by noradrenaline, whereas an increase in the output of glucose was found

The bile flow decreased immediately after the noradrenaline infusion was started but within 15—30 min the bile flow returned to the control level The biliary excretion of bile acids and the biliary recovery of ICG was not significantly changed by noradrenaline The EHBF was unchanged by infusion of noradrenaline at rates of 0 5 and 1 0 μg/kg/min and decreased when the infusion rate was 2 0 and 5 0 μg/kg/min (15 and 23 % resp) The arterial blood pressure increased in proportion to the infusion rate of noradrenaline indicating a correlation between the noradrenaline dose and the relative change in splanchnic vascular resistance

It is concluded that noradrenaline in the present doses does not cause a decrease in the functional capacity of the liver, and consequently the number of perfused sinusoids remains unaltered

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D 15

The Immediate Effects of Ligation of a Liver Lobe

By J A. LARSEN *Institute of Physiology University of Aarhus, Denmark*

In experiments on 21 chloralose anesthetized cats the liver function was followed before and after ligation of the left liver lobe By this procedure the amount of liver tissue was reduced 29 % A corresponding decrease in bile flow was observed the correlation coefficient being 0 91 The reduction in the total elimination rate of ethanol did not correlate well with the reduction in liver tissue In general the reduction was smaller than expected and in 2 expts there was no change in elimination rate When calculated as μmol/100 g liver/min ligation caused a significant increase in elimination rate from 118 to 129 The biliary excretion of Indocyanine Green was 96 % of the amount infused in the control period and changed to 85 % after ligation

In addition to arterial blood pressure the portal pressure was measured in 6 cases together with registration of the portal and hepatic arterial flow Ligation caused a permanent increase in hepatic arterial resistance whereas only a small and sometimes temporary increase in portal resistance was noticed Ligation caused a decrease in hepatic arterial flow (21—13 ml/kg/min) but portal flow was unchanged (19 ml/kg/min)

In conclusion ligation of a liver lobe is accompanied by a corresponding decrease in bile flow but only a small decrease in the excretory capacity Judged from the total elimination rate of ethanol the liver may compensate for the reduction in liver cell mass by increasing the metabolic capacity The mechanism of this compensation is unknown

D 16

A Technique for Rapid Separation of Isolated Fat Cells from their Incubation Medium

By S GAMMELTOFT J GLIEMANN J VINTEN and K ØSTERLIND *Institute of Medical Physiology C University of Copenhagen Denmark*

The lipid stores of collagenase isolated rat fat cells constitute about 95 per cent of the cell volume and a volume of about 30 per cent of this is usually trapped between packed cells Therefore the free fat cell preparation has not been well suited for the study of factors which are distributed in the intracellular waterspace The present communication describes a method whereby free fat cells are separated rapidly and almost completely from the incubation medium

Free fat cells were incubated in homogeneous suspensions at 37° C on magnetic stirrers 300 μl aliquots were transferred to polyethylene tubes and the cells were centrifuged for 15 s through a layer of oil with a specific gravity between that of the cells (ca 0 90) and that of the buffer (ca 1 02) Fat cells were recovered quantu

tively above the oil, and ^3H_2O, ^{3}H-inulin, 3 O methyl ^{14}C glucose and ^{125}I insulin did not dissolve in the oil to any significant extent After passage through the oil, the inulinspace of packed cells was about 1 0 μl/100 μl and the total waterspace was about 2 6 μl/100 μl The coefficient of variation for the intracellular waterspace measured on the same pool of cells was 8 per cent

The potentials of the method are illustrated by the following measurements ^{125}I insulin (20 μU/ml) was bound to fat cells and partially displaced by the addition of unlabelled insulin (10^5 μU/ml) Insulin stimulated uptake from the extracellular medium of 3 O methyl-^{14}C-glucose, $^{42}K^+$, $^{22}Na^+$ and $^{45}Ca^{++}$

D 17

Changes in Serum Enzyme Levels after 90 km Cross-Country Skiing

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Physical exertion has been shown to induce increases in serum enzyme levels (Hunter and Critz 1971 Schwartz *et al* 1971) Most investigations have been carried out in connection with exercise of relatively short duration, and little is known about the influence of prolonged heavy exercise, such as *e g* long distance cross country skiing The purpose of this investigation was to study the changes in serum levels of creatine phosphokinase (CPK), glutamic oxalacetic transaminase (GOT), lactate dehydrogenase (LDH) LDH isoenzymes (LDH-1 to -5) and ornithine carbamyl transferase (OCT) in association with a 90 km cross country skiing competition known as the Grenader race

Fortyone male participants, aged 21 to 54, volunteered as subjects in the 1971 Grenader race They required from 6 1/2 to 10 1/2 h to complete the distance, under conditions of heavy snowfall and temperature around 0° C Venous blood samples were obtained before and immediately upon completion of the race Seventeen of the subjects returned to the laboratory for blood sampling on the first, third and fifth day following the competition

Highly significant rises were observed in CPK, GOT, total LDH and LDH 4 and -5, whereas only minor changes were noted in LDH-1, -2, -3, and OCT Immediately after the race the CPK activity was increased to 3 times the level prior to the race, on the day following the competition it was further increased to 6 times the initial level (to 548 U/l, at 30° C) Similarly at the finish of the race, the activity of GOT was increased to about 1 1/2 times the initial value, and reached its peak value of approximately 2 1/2 times the initial level on the following day (49 U/l) The CPK and GOT activity did not return to the initial level until the fifth day after the race while the LDH activity and isoenzyme fractions of LDH were normalized already on the day following the competition

The main source of CPK is skeletal and cardiac muscle whereas OCT originates in liver Since only minor changes were observed in OCT and in the activity of the isoenzyme fractions found in cardiac muscle, erythrocytes, kidney and lung (LDH-1, 2, 3) it seems reasonable to infer that striated muscle was the major source of enzyme release The prolonged elevations in serum levels of CPK and GOT may indicate that the organism is not fully restituted until at least 5 days following this type of severe muscular exertion

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D 18

Active State in Vascular Smooth Muscle

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According to a mechanical model of striated muscle proposed by Hill (1938) the contractile element is coupled in series with a passive elastic element An isometric contraction then always involves some internal shortening of the contractile element as it stretches the series elastic component A further consequence of this arrangement and of the force velocity relationship is that the isometric force recorded during a twitch does not give a correct picture of the time course and the intensity of the chemo-mechanical transduction the active state The isotonic quick release method is one of the experimental procedures used to study the active state in twitches of skeletal or cardiac muscle (*e g* Edman and Nilsson 1968)

Smooth muscle is not easily subjected to such analysis due to its irregular and unpredictable behaviour The portal vein of the rat which shows spontaneous contractions of quite variable configuration in standard Krebs solution was found to develop a highly regular electrical and mechanical activity when exposed to simultaneous increase in [K] and [Ca] Fig 1 A) Quick release experiments were performed under these conditions (Fig 1 B by using a set up which allowed simultaneous recordings of force and shortening The early rate of shortening under a small load which followed the immediate recoil of the series elastic element was taken as an indicator of the intensity of the active state at the respective stages of the uniform contraction *cf* Edman and Nilsson 1968) Fig 1 C shows the time course of the active state thus obtained together with a recording of isometric force

The results indicate that concepts concerning the mechanical properties of striated muscle are applicable in principle also to vascular smooth muscle The regular activity exemplified by Fig 1 A may provide a useful model for a quantitative approach to problems of electro mechanical coupling in this tissue

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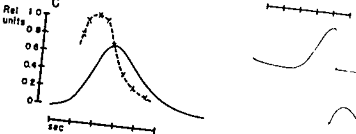


Fig 1 *A* Sucrose-gap recording of electrical (above) and mechanical (below) activity of an isolated portal vein in a Krebs solution, containing 25 mM K^+ and 17 5 mM Ca^{++} *B* A series of quick releases performed under such ionic conditions with recording of force (above) and shortening (below) The shortening of the muscle in response to a sudden reduction in force consists of a fast immediate phase, mainly due to series-elastic recoil, and a second phase, due to shortening of the contractile element Rate of shortening 100 ms after release was taken as an indicator of the intensity of the active state *C* Intensity of the active state in relative units (crosses) at different stages of the isometric contracion (full line)

D 19

Changes in the Tissue Levels of cAMP in the Gall-Bladder and the Sphincter of Oddi Induced by the C-terminal Octapeptide of Cholecystokinin

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Cholecystokinin (CCK) and its C-terminal octapeptide (C8-CCK) contract the gall bladder and relax the sphincter of Oddi by direct effects on the smooth muscle cells (Hedner 1970, Persson 1971). These actions are not affected by adrenoceptor blocking drugs or by atropine.

Cyclic adenosine 3',5'-monophosphate (cAMP) has been demonstrated to stimulate the activity of a microsomal calcium binding mechanism in smooth muscle (Andersson *et al.* to be published). Thereby cAMP may influence the intracellular concentration of the free calcium ions necessary for contraction. It has been suggested (Amer 1969) that variations in the intracellular cAMP content may be involved in contraction and relaxation of the gall bladder. In order to test this suggestion, changes in cAMP concentration and phosphodiesterase (PDE) activity induced by C8-CCK in the gall bladder and the sphincter of Oddi were investigated in the present study. Gall bladder strips from guinea pigs and longitudinal preparations of sphincter of Oddi from cat were mounted in organ baths containing Krebs solution, gassed with carbogene and kept at 37° C. Isometric tension was recorded. C8-CCK ($1-2\times10^{-8}$ g/ml) was added to the bath and the metabolic changes were measured after about 50 and 180 seconds. cAMP was determined according to Kakiuchi and Rall (1968) and PDE activity according to Kukovetz and Pöch (1970). Twenty seconds after the administration of C8-CCK, just as the tension began to develop in the gall bladder strip, there was a 67 per cent decrease of the cAMP content and an almost 50 per cent increase of the PDE activity ($P<0.02$). After 180 s when the tension response had reached its maximum, these changes were still present, though less pronounced.

When the relaxation of the sphincter of Oddi induced by C8-CCK was fully developed after about 3 min, the cAMP level was increased by 41 per cent ($P<0.01$). In two preliminary experiments the PDE activity was increased by 45 and 47 per cent respectively after 3 min 30 s after the administration of C8-CCK; the PDE activity was increased by 7—20 per cent in the sphincter of Oddi (4 expts).

From these results it can be concluded that C8-CCK reduces the cAMP content in the gall bladder by activation of PDE. In the sphincter of Oddi the cAMP content increased after C8-CCK. This cannot be produced by an effect on the PDE activity but might be explained by stimulation of adenylcyclase.

It is suggested that the metabolic effects demonstrated are of importance for the initiation of the mechanical responses to C8-CCK in the gall-bladder and in the sphincter of Oddi

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D 20

Ion Efflux in Denervated Frog Muscle

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Measurements of concentrations of monovalent and divalent cations in denervated rat muscle show a relative increase in calcium content and only a small decrease in total potassium The calcium "gain" is evident only after a week and apparently begins shortly after denervation (Hines and Knowlton 1933) Sodium was not analyzed, and although some indirect measurements are available (Guth 1968), cation efflux determination in denervated muscle was found to be of relevance

) Chemosensitivity changes, in particular to iontophoretically applied acetylcholine, occur in vertebrate muscle in response to denervation In mammalia, changes at the membrane seem to originate at the endplate region and to spread out from there and to complete within one or two weeks (Axelsson and Thesleff 1959)

Denervated muscles and controls (tibialis anticus longus) from frogs kept at 4° C were dissected after 4 days and measured with respect to sodium extrusion in normal saline Uptake of denervated mucle is less than that of the control at room temperature, but any strict dependence on weight atrophy could not be detected With respect to sodium efflux, no significant difference occurred between normal and denervated muscle after approximately 90 min, and rate constants were comparable for the slow components of the efflux curves at 5 pM Na/cm^2 s

Tibialis anticus muscles were incubated with ^{45}Ca 4 days from denervation Calcium extrusion at room temperature attains relatively higher values in denervated than non-denervated muscle when tested by means of 5-hydroxytryptamine (10^{-4} M) at 40 min length 3 h from the start of the washout This phenomenon occurred within the first week of denervation, in the experiments made so far, hardly any difference between operated muscle and control was found on the eighth day, whereas calcium could be sampled *from denervated muscle in relative excess* of normal on the second day

Although 5 HT has a pronounced effect on calcium efflux, the absolute values of extruded calcium of both normal and denervated muscle proved to be liable to some variation. Sodium extrusion does apparently not depend on presence of 5 hydroxytryptamine

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C 1

The Stimulating and Inhibitory Effect of Choline on Decamethonium Uptake by Rat Kidney Cortex Slices

By J Holm, *Department of Pharmacology, University of Copenhagen, Denmark*

The existence of a specialized transport system for the monoquaternary ammonium compound choline has been established in rat kidney cortex slices (Sung and Johnstone 1965, 1969) We have investigated whether the polymethylene-bis-quaternary ammonium compound decamethonium, which is concentrated in rat kidney cortex slices (Mc Isaac 1969) uses this transport system

Kidney slices were incubated in Krebs-Ringer bicarbonate medium (37° C, pH 7 4, aeration 95 % O_2 and 5 % CO_2) containing 2×10^{-6} M ^{14}C-decamethonium and ^{14}C slice to medium concentration ratio (S/M ratio) was measured

Table I shows that decamethonium uptake is increased in the presence of 10^{-3} M and 3×10^{-3} M choline whereas 5×10^{-2} M choline inhibits uptake Initial decamethonium uptake (5 min) was increased (23 ± 5 per cent, $p<0\ 005$) from 1 00 ± 0 03 to 1 22 ± 0 03 (mean ± S E n = 9), when slices were incubated (1 1/2 h) with 3×10^{-3} M choline (without decamethonium) before transfer to a choline free medium containing decamethonium These results suggest that stimulation is related the presence of choline in the slices Slices were incubated (1 h) with 2×10^{-6} M decamethonium before transfer to another medium containing 2×10^{-6} M unlabelled decamethonium No ^{14}C-efflux (1/2 h) occurred into the latter medium, which means that the increased net uptake (influx—efflux) cannot be attributed to inhibition of decamethonium efflux by internal choline

The stimulation phenomenon is interpreted as an example of substrate facilitated carrier transport which should mean that choline efflux accelerates decamethonium influx This interpretation is consistent with the conclusion from the experiments on mutual inhibition that choline and decamethonium share a common transport mechanism

TABLE I Effect of choline on 1 h uptake (S/M ratio) of ^{14}C-decamethonium (2×10^{-6} M) by rat kidney cortex slices [a]

Choline conc (M)	Number of expts	S/M ratio		Difference (%)
		Control	Choline added	
1×10^{-3}	6	5 3 ± 0 1	9 2 ± 0 4	+77 ± 10[b]
3×10^{-3}	9	4 8 ± 0 2	8 9 ± 0 4	+89 ± 11[b]
5×10^{-2}	9	6 0 ± 0 2	4 2 ± 0 1	−30 ± 3[b]

[a] Kidney slices from each animal (distributed in two test tubes) were incubated either with or without (control) choline Mean values ± S E are given

[b] Mean percentage values differ significantly from zero $P < 0\ 001$

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C 2

Amino Acid Transport in Isolated Rat Ovaries Competitive Inhibition between Neutral Amino Acids and Effect of Follicle Stimulating Hormone

By K Ahren T Hillensjo and G Selstam *Department of Physiology, University of Goteborg, Sweden*

Earlier work (Ahren and Kostvo 1963 Ahren *et al* 1967) has shown that a single iv injection of follicle stimulating hormone (FSH) enhances the *in vitro* accumulation of the non utilizable amino acid analogues α aminoisobutyric acid (AIB) and 1 aminocyclopentanecarboxylic acid (cycloleucine) It has also been demonstrated that the kinetics of the two amino acid analogues differ markedly in the ovary The present study is a further investigation of ovarian amino acid transport in order to analyse the mechanism of the FSH effect

Isolated ovaries from 23 day old rats were used The ovaries were incubated in Krebs bicarbonate buffer containing 5 5 mM glucose 0 1 mM AIB-^{14}C or cycloleucine ^{14}C with or without addition of an unlabelled normal neutral amino acid (10 mM) to study competitive inhibition between each amino acid and the analogue The distribution ratio of the labelled analogue (cpm per ml intracellular water/cpm per ml medium) was measured after 2 h of incubation The AIB uptake was only slightly inhibited by the following amino acids in sequence tryptophan (try), tyrosine (tyr) phenylalanine (phe) leucine (leu) valine (val) and isoleucine (ileu) where val was the most powerful inhibitor If the control value of AIB ^{14}C distribution ratio being in the range of 9—12 in each experiment is set to 100 % and a complete inhibition of this distribution to 0 % the values for his group lie between 100 % and 60 % Another group of amino acids showed strong inhibition namely in sequence glycine (gly) glutamine (gln) asparagine (asn) serine (ser) alanine (ala) and methionine (meth) varying from 40 % for gln to 20 % for meth

Christensen has suggested and named several types of transport systems for amino acids (*e g* Oxender and Christensen 1963) Thus in the Ehrlich ascites cell two distinct mediating systems for transp rt of neutral amino acids A and L-systems have been characterized mainly according to their maximum distribution ratio stereospecificity sensitivity to alkali metal ions and temperature The first group of amino acids which in our study gave slight inhibition correlates with the L-system and the second group with the A system

The uptake of cycloleucine ^{14}C could also be inhibited by various neutral amino acids No definite grouping could however be seen the amino acids belonging to the A and L systems produced roughly the same degree of inhibition Most of th

were in the range of 55 % to 35 % When AIB and cycloleucine were tested against each other it was found that cycloleucine inhibited AIB-^{14}C uptake to a greater extent than AIB inhibited cycloleucine ^{14}C uptake This suggests that in the ovary cycloleucine is transported both via the A and L systems while AIB is mediated more exclusively via the A system

In another sereis of experiments the effect of FSH was studied A dose of 250 μg of FSH (NIH FSH S8) was injected i v to the rats 30 min before sacrifice This resulted in a stimulation of AIB ^{14}C uptake *in vitro* as earlier has been shown When leu or val was added to the incubation medium no effect of FSH on AIB uptake was seen Ala or ser on the other hand did not eliminate the stimulatory effect of FSH Thus the patterns of competition for the amino acids was changed after FSH treatment These results are compatible with the hypothesis that FSH acts differently on the A and the L system A metabolic adjustment in the ovarian cells *cannot however yet be excluded*

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C 3

e Effect of CO on the Permeability of the Blood Brain Barrier to Cr^{51}-EDTA nd Na $^{+}$

By SOREN CLAUS SORENSEN *Institute of Medical Physiology Dept A University of Copenhagen Denmark*

The *diffusion of hydrophilic substances between blood and interstitial* fluid is impeded in the brain as compared with other tissues which has led to the concept of a blood brain barrier A controversy however remains regarding the location of this barrier Electron microscopy studies have revealed that proteinaceous substances cannot pass the endothelial lining in the cerebral vasculature because cerebral endothelial cells are connected with tight junctions which encompass the cells in their entire circumference but it is not known whether these junctions also are impervious to small molecules such as alkali metal ions This study was performed to examine the latter question

The permeability of the blood brain barrier to albumin and sucrose is increased when high concentrations of CO_2 are administered in the inspired air (Clemedson *et al* 1958 Cutler and Barlow 1966 Cameron *et al* 1970) This suggests that the tight junctions may be opened up in hypercapnia We would similarly predict an increased permeability of the blood brain barrier to sodium in hypercapnia if these junctions also restrict the diffusion of sodium Accordingly in the present study we compared in anesthetized rats the effect of 25 % CO on the permeability of the

Table 1 The uptake of Cr^{51} EDTA and Na^{24} in rat brain The blood concentration of the isotope was maintained constant for one hour before the brain was removed The concentration of isotope in the brain samples is expressed as $\frac{\text{cpm/g brain}}{\text{cpm/g plasma } H_2O} \times 100$

		Hemisphere	Cerebellum	Pons + medulla obl
Cr^{51} EDTA	Control n = 12	1 42 ± 0 21	1 80 ± 0 24	1 75 ± 0 11
	25 % CO_2 n = 12	[1]3 23 ± 0 65	[1]3 93 ± 0 84	[2]2 77 ± 0 32
Na^{24}	Control n = 12	13 39 ± 1 12	14 71 ± 1 05	14 31 ± 1 28
	25 % CO_2 n = 12	15 87 ± 1 15	15 33 ± 1 08	12 94 ± 0 77

[1] Significantly different from control value $p < 0.025$ (students t test)
[2] Significantly different from control value $p < 0.010$ (students t test)

blood brain barrier to Cr^{51} EDTA (MW = 359) and Na^{24} by measuring the concentrations of the 2 isotopes in brain samples after their plasma concentrations had been maintained constant for 1 h The concentrations of isotopes in the brain samples were corrected for the intravascular amount of isotopes using I^{131} albumin as the plasma tracer

During hypercapnia the uptake of Cr^{51} EDTA in the brain was significantly increased but the uptake of Na^{24} was not altered The results show that the mechanism which is responsible for the increased uptake of Cr^{51} EDTA during hypercapnia does not affect the uptake of Na^{24} Assuming that the effect of hypercapnia on the uptake of Cr^{51} EDTA is due to an opening of the tight junctions between endothelial cells the results suggest that the diffusion of sodium between plasma and brain interstitial fluid is not restricted by these junctions It must be emphasized that the conclusion is based on the assumption that the major fraction of sodium exchange across the blood brain barrier is by diffusion through intercellular channels This assumption has recently been validated by Davson and Segal (1970)

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C 6

Glutamic Acid Sensitivity of Hippocampal Pyramidal Cell Dendrites

By J D DUDAR *Institute of Neurophysiology University of Oslo Norway*

Glutamic acid is a possible central excitatory synaptic transmitter However its excitatory action may be non specific since glutamate excites all types of neurones (Curtis and Crawford 1969) one alternative being that it acts by promoting the entry of Na ions into the cell (Krnjevic 1970) The hippocampal slice (Skrede and Westgaard 1971) is a promising *in vitro* preparation in which to study putative transmitters because the anatomy allows ready access to and visualization of several identifiable synaptic regions as well as the cell body layer

Slices of guinea pig hippocampus cut transversely to the long axis were used to study the effects of glutamic acid on spontaneously active CA1 pyramidal cells The activity of the CA1 pyramids was recorded through 1 5 M NaCl pipettes (1—4 MΩ) while 1 M Na glutamate (pH 7 0 2 5—5 MΩ) was administered iontophoretically from a second movable pipette

Glutamate was very effective in exciting CA1 pyramids when ejected close to the recording site in the cell body layer which agrees with the findings of Biscoe and Straughan (1966) in the intact animal In addition glutamate could also excite the CA1 pyramids when ejected into the stratum radiatum at distances 100—400 μ normal to the CA1 pyramids The excitation of the CA1 pyramids was confined to a strip normal to the cell body layer corresponding to the direction of the apical dendrites Moving the glutamate pipette 100 μ to either side of this strip or to sites lay in a semicircle about the recording site failed to excite the CA1 pyramids yen with 2 5 times the current needed when at the optimal site Cathodal currents ejected from NaCl pipettes in the dendritic field did not excite the CA1 pyramids until the current was 6 times larger than the glutamate current Furthermore glutamate was more effective when ejected within the slice than when on the surface Glutamate excitation of the CA1 pyramids from the dendritic synaptic area in the stratum radiatum was observable within 300 ms

The localized nature of the glutamate response and its short latency suggest that glutamate is acting upon the synapses in the dendritic field rather than diffusing down to the soma and acting there The implication is that glutamate requires synapses in order to depolarize neurones even though this effect may be through a non specific mechanism

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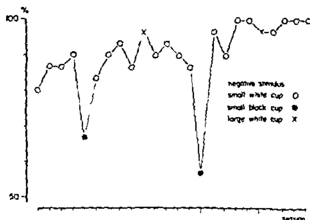
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C 7

Visual Discrimination after Total Neonatal Removal of the Neocortex in the Cat

By Kerstin Norrsell and Ulf Norrsell, *Department of Physiology and Department of Ophthalmology, University of Göteborg, Sweden*

Fig 1 Percentage of correct responses in 25 consecutive sessions of visual discrimination performed by a cat in a T maze (30 trials/session) Both cerebral hemispheres had been removed within two weeks after birth, one and a half year before the experiments The positive stimulus was a large black cup in all sessions The negative stimulus was a small white cup in all sessions except 5 10 15 and 20 In sessions 5 and 15 the stimulus was a small black cup and in sessions 10 and 20 a large, white cup



Removal of the striate cortex in the cat appears to cause a quantitative rather than qualitative impairment of vision especially after lesions performed neonatally (Doty 1961, Tucker *et al* 1968) A loss of pattern vision has been reported if the lesions include the suprasylvian and ectosylvian areas as well (Doty 1961 *cf*, however, Meyer 1963) The residue of vision after total removal of the neocortex in the cat is little known Visually guided spontaneous behaviour has been observed after that lesion performed neonatally (Norrsell and Norrsell 1970) and it has now been investigated whether such cats will utilize vision in a learning situation

Cats with both cerebral hemispheres removed neonatally have been trained in a T maze The positive stimulus was a large black cup (145×55 mm) containing food and the negative stimulus a small white, empty cup (80×30 mm) The cats were starved for 24 h before each experimental session and the stimuli were interchanged according to Gellerman s series Fig 1 shows 25 consecutive sessions for the best cat, commencing one month after the training had started Learning was still improving and reached a 100 % level at the end of the period The negative stimulus was altered in sample sessions and when changed to a small black cup of the same size as the white standard negative stimulus the performance dropped to slightly above chance level The performance was not changed by introducing a large white cup as negative stimulus (size of standard positive stimulus) The cue for the discrimination hence appears to be brightness rather than form Olfactory cues were excluded in control experiments

Primitive behavior based on learning has been shown in animals without tex in classical and instrumental conditioning situations (*cf* *e g* Pol

Zelions 1930, Bromiley 1948) The ability of the present decorticate animals to learn the more complex behaviour of stimulus discrimination coupled to an adapted motor performance is probably due to the lesions having been inflicted neonatally

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C 8

Afferents to the Dorsal Column Nuclei from the Dorsolateral Funiculus of the Spinal Cord

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Dart and Gordon (1970) recently showed that units in both gracile and cuneate nuclei in the cat could be activated from the ipsilateral dorsolateral funiculus (DLF) Several of these units had typical cell spikes and often showed characteristics suggesting possible monosynaptic activation Lesions indicated that the pathway for this excitatory input runs through the DLF

The present experimental neuroanatomical study confirms this indication and extends the knowledge as to details of the pathway The method of successive degeneration (Sherrington and Laslett 1903) was adopted in 2 cats The DLF was sectioned unilaterally at T 10—11 and at C 7 respectively 13 months following bilateral division of the dorsal columns at C 4—5 Five to six days later the animals were killed under deep anesthesia by intravital perfusion with formaldehyde Frozen serial sections from the dorsal column nuclei treated according to the silver methods of Nauta (1957 and Fink and Heimer (1967 showed an abundant fibre degeneration in the gracile nucleus ipsilateral to the DLF lesion in both cats The fibres seemed to enter the nucleus from its ventral side after coursing mediodorsally from the DLF In the cat with the DLF lesion at C 7 degenerating fibres were also found in the ipsilateral cuneate nucleus

In three additional cats DLF lesions were performed at C 1 C 4—5 and L 4 respectively These animals were killed 5—6 days postoperatively Degenerating fibres were found in the gracile nucleus in all these cases but only in a restricted number in the cat with the L 4 lesion No degenerating fibres were found in the cuneate nucleus following the L 4 lesion.

The findings show that fibres from the DLF are given off to and apparently terminate in the gracile and cuneate nuclei

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C 9

Concentrations of Free Amino Acids and Inorganic Ions in Red Blood Cells of Flounder (Pleuronectes flesus L) during Adaptation to Fresh Water

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When the euryhaline flounder (*Pleuronectes flesus L*) is adapted to fresh water, the plasma osmolality of the fish is reduced The reduction in plasma osmolality is usually accompanied by a lowered water content in the red blood cells and also by a decreased intracellular concentration of ninhydrin positive substances (NPS) These observations have been interpreted as indicating that no osmotic swelling of the red blood cells occurs when plasma osmolality is reduced and that there is an active regulation of the cell volume in which the erythrocyte NPS take part (Fugelli and Lange 1965) It has further been shown that the amino acids taurine and gamma amino butyric acid (GABA) are the quantitatively dominating ninhydrin positive substance in erythrocytes of flounder (Fugelli 1970) Exact quantitative examinations of the concentrations of amino acids and inorganic ions during periods with lowered plasma osmolality have as yet not been made

In the present experiments flounders were adapted to fresh water for a period varying from 5 to 29 days during which the plasma osmolality was reduced From the observed data (Table I) it can be seen that the concentration of sodium seems to vary inversely with plasma osmolality while potassium and chloride decrease with

Table I Mean concentrations of inorganic ions and amino acids (mmol/kg intracellular water ±S D) in red blood cells of flounder (*Pleuronectes flesus* L) related to plasma osmolality (mOsm) Number of fishes analysed given in parantheses

Osmolality group	Sodium	Potassium	Chloride	Taurine	GABA
350—320	19 6±3 4 (3)	148 5±5 6 (6)	74 5±3 0 (8)	32 8±7 3 (5)	15 0 ±1 9 (5)
319—300	29 0±2 1 (3)	137 7±0 6 (3)	71 6±1 3 (4)	27 8±5 2 (4)	12 2±4 0 (4)
299—280	28 6±12 3 (3)	145 2±13 2 (4)	67 5±10 2 (5)	21 1±7 6 (4)	6 7 ±1 8 (4)
279—260	24 6 (1)	139 0±17 8 (2)	60 3±5 4 (2)	11 3±2 0 (2)	5 1±2 8 (2)

plasma osmolality, but these changes are statistically insignificant The sum of inorganic ion concentrations varies only very slightly when plasma osmolality decreases The concentrations of taurine and GABA, however, decrease markedly with decreasing osmolality The change in concentration of taurine and GABA accounts for about 50 per cent of the change is osmolality The remaining 50 per cent must therefore be due to changes in other solutes

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C 10

The Relationship between the Fluxes of Hydrogen and Oxygen Isotopes of Labelled Water and the Water Flux across a Hydrophobic Membrane

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Both deuterated water and tritiated water are commonly used to monitor the movements of water in biological systems The inherent uncertainties which can arise from the significant differences in the atomic masses of these three isotopic forms of water, and from differences is symmetry of the molecelues, seem widely appreciated and indeed it is usual to adjust the measured isotope fluxes in accordance with the data of Wang, Robinson and Edelman (1953) on the diffusion coefficients of water variously labelled with deuterium, tritium and ^{18}O When water labelled with a hydrogen isotope is used there is a further complication which seems to be less well acknowledged the water protons may exchange with the tissue protons, or more particularly, with protons in membranes Disregard of this consideration may not introduce an error in the interpretation of experiments made under steady state conditions, provided that the proton exchanges are limited to simple jumps between fixed membrane sites and closely adjacent water molecules If however the membrane acts as a bridge or pathway for protons linking distant water molecules the isotope flux measurements will not reflect water movements The use of ^{18}O seems to be generally regarded as unobjectionable but we present some results here which indicate the lack of generality of this assumption

We have made some studies on hydrophobic membrane (Gelman VF—6) which is impermeable to water (bulk flow) provided the hydrostatic pressure is below about 1 atm This membrane was mounted between 2 compartments containing well stirred volumes of water or aqueous solutions in a thermosted glass diffusion chamber The fluxes of deuterium, tritium and ^{18}O, introduced as isotopically labelled water, across the membrane were examined in the absence of any hydrostatic or

osmotic difference The isotopes were measured by the appropriate gravimetric, li quid scintillation or mass spectrometric techniques The membrane in addition to be ing apparently impermeable to water even in the presence of a small hydrostatic pressure gradient was also impermeable with a sucrose induced osmotic gradient (0 5 atm) in the sense that no net movement of water was observed over 15 days Both hydrogen isotopes and ^{18}O however traversed the membrane at approximate ly the same rates In this case when the system contained only water the net water flux should be zero but clearly from the isotope data the unidirectional fluxes are not zero

The membrane while thus allowing the passage of water molecules does not permit a net water flow in the presence of an apparent osmotic gradient Thermodynamically this seems to mean that even with different solute concentrations the effective chemical potentials on both sides of the membrane must be equal and the possible mechanisms underlying this phenomenon will be discussed

The results exemplify a fallacy in generally assuming that isotope fluxes of labell ed water reflect net water movements

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C 11

Effects of Lanthanum and of Oligomycin and Cyanide on Release of Vasopressin from Isolated Rat Neurohypophyses

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In the neurohypophysis as in many other endocrine systems calcium has been shown to be essential in the excitation secretion coupling In several other s stems with calcium transport lanthanum has been shown to interfere with this transport (see *e g* Miledi 1971) It has been suggested that metabolic energy is involved in one of the steps that link stimulation of the neurohypophysis and the release of vasopressin (Douglas *et al* 1965) Baker *et al* (1971) have recently shown that oligomycin and cyanide in giant axons cause a marked increase in the intracellular calcium ion concentration

In the present experiments the effect of the presence of lanthanum in the incuba tion medium (modified McIlwain Rodnight medium with 50 mM Tris) of isol ed hemilobes of rat neurohypophyses was studied while they were resting or stim ed by a high potassium concentration (56 mM) Vasopressin release to the med m was measured by pressor assay Lanthanum in a concentration of 0 4 mM ca ed a reduction of the unstimulated release and of the potassium induced release 4 mM lanthanum completely abolished the potassium induced release (see Fig 1 M S D are given)

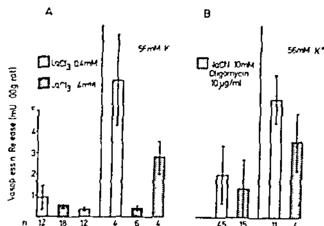


Fig 1

In another series of experiments we have tested the effect of a combination of 10 mg/l of oligomycin with 10 mM sodium cyanide in an incubation medium on the release of vasopressin described above. Cyanide and oligomycin caused a slight de crease in the release from unstimulated neural lobes The amount released after stimulation was reduced by about 35 % (Fig 1) Thus, some release can take *place when synthesis and utilization of ATP* are inhibited

Apparently lanthanum interferes with calcium transport also in the present system It would appear that the release of vasopressin must involve cell membrane

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C 12

Enzyme Activity and Fiber Composition in Human Skeletal Muscle

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Animal studies have demonstrated that training can increase the activities of some Krebs cycle enzymes, the capacity for fat oxidation, and the mitochondrial protein concentration of skeletal muscle (Holloszy *et al*, 1971) Some evidence indicates that training can elicit similar changes in *human skeletal muscle* (*Kiessling, Piehl* and Lundqvist 1971) In this study the metabolic and histochemical characteristics of

TABLE I

Subjects	Muscle groups	$\dot{V}_{O_2}$ max ml/(kg × min)	Fibers STR %	Enzymes SDH μM/(g × min)	PFK
Untrained	arm	—	46 1±6 8	3 6±0 4	21 6±3 7
n = 12, 27 years	leg	43 (32—53)	36 8±5 0	4 3±0 6	25 3±2 1
Bicyclist	arm	—	50 7±4 4	6 1±0 2	24 0±4 0
n = 4, 24 years	leg	68 (64—75)	61 4±5 9	11 0±1 0	23 4±1 4
Canoers	arm	55 (51—60)	58 4±3 8	7 9±0 6	25 1±6 8
n = 4, 26 years	leg	57 (55—58)	61 4±6 2	5 8±0 9	22 2±4 7
Runners	leg	72 (61—82)	58 9±3 7	6 4±0 5	20 1±2 5
n = 8, 23 years					
Swimmers	arm	—	74 3±5 9	8 6±0 7	22 7±0 4
n = 5, 21 years	leg	71 (64—74)	57 9±9 3	7 6±0 5	29 3±0 4
Weightlifters	arm	33 (27—40)	52 6±7 7	2 6±0 4	21 1±4 8
n = 4, 25 years	leg	42 (32—55)	46 1±10 5	3 0±0 3	24 7±1 7

Mean value (±S E) for succinic dehydrogenase (SDH), phosphofructokinase (PFK) and fiber composition (STR — slow twitch red) in muscle samples taken from the lateral portion of the thigh and the deltoid muscle The percentage of fast twitch fibers (FTW) is obtained by subtracting the percent of STR from 100 Included is also maximal oxygen uptake of the studied groups

skeletal muscle of sedentary as well as world champion endurance athletes were examined Aerobic and anaerobic potentials of biopsy samples from the vastus lateralis and *deltiod muscles were estimated from succinic dehydrogenase (SDH)* and phosphofructokinase (PFK) activities respectively Muscle fibers were classified on the basis of myosin ATPase and DPNH diaphorase activities The intramusclar distribution of oxidative capacity was estimated from the DPNH diaphorase activity Maximal oxygen uptake was determined for all subjects during leg work and also during arm work for some groups

The results (Table I) show that SDH activity was highest in muscles that participated in endurance exercise (biocyclists and swimmers) and lowest in weight lifters and untrained subjects Differences between SDH activities of the arm and leg muscles suggest a specific local training response The only difference in PFK activity was that of the leg muscle of the swimmers which was higher than all other groups

Fast twitch white (FTW) and slow twitch red (STR) fibres were identified in the muscles examined (Barnard *et al* 1971) The FTW fibers were most common in the muscles of the untrained subjects Greater percentage of STR fibers w

found in the muscles of the athletes that possessed high SDH activities, but a large range for the percentage distribution of fibers was observed for all studied groups DPNH diaphorase activities revealed that differences in oxidative capacity resulted from changes in both fibre types The oxidative capacity of the FTW fibres of bicyclists, canoers, runners and swimmers appeared to be as great as that of the STR fibers of the untrained groups Maximal oxygen uptake was not directly related to the oxidative potential of the muscles studied

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C 13

Free Fatty Acids and Myocardial Function during Ischemia

By J K KJEKSHUS and O D MJOS, *Institute for Experimental Medical Research, University of Oslo, Ullevål Hospital, Norway*

Elevation of plasma concentrations of free fatty acids (FFA) has been shown to increase myocardial oxygen consumption without influencing mechanical performance in normal hearts (MJOS 1971) Accordingly the purpose of this study was to determine whether FFA would modify mechanical performance when oxygen supply to the heart is limited Left coronary blood flow was reduced by gradual clamping of a shunt from the left carotid artery until moderate ventricular dilatation supervened Left ventricular systolic pressure (LVSP) its maximal rate of rise (dP/dt) and stroke volume (SV) remained virtually constant The ischemia resulted in a decrease in myocardial oxygen consumption (MVO_2) from 9 7 ± 1 1 ml/min to 7 9 ± 0 8 ml/min and myocardial lactate uptake was reduced or reversed to excretion Increasing the plasma concentrations of FFA from 359 ± 47 μEq/l to 3688 ± 520 μEq/l by i v infusion of a triglyceride emulsion and heparin resulted in further ventricular dilatation accompanied by increased excretion of lactate The ventricular decompensation and enhancement of anaerobic myocardial metabolism associated with increased uptake of FFA was not related to changes in coronary flow, MVO_2 or LVSP dP/dt and SV were unchanged or slightly reduced Intravenous infusion of glucose/insulin which lowered plasma concentrations of FFA reversed ventricular dilatation and lactate excretion The data are consistent with the hypothesis that FFA play a significant role in increasing myocardial oxygen requirement and thereby promote depression of myocardial contractility of the oxygen limited heart

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C 14

Termination Areas of Climbing Fibre Paths in Paramedian Lobule

By J D Cooke, O Oscarsson and B Sjölund *Institute of Physiology University of Lund, Sweden*

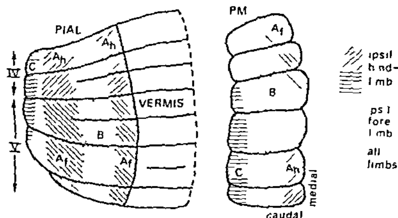


Fig 1 Termination zones of climbing fibre paths in pars intermedia of lobules IV and V of anterior lobe (PIAL) and in paramedian lobule (PM) Spinal paths classified according to Larsson *et al* (1969) contributing to each zone are tabulated

Armstrong Harvey and Schild (1971) demonstrated that some climbing fibre paths branch to innervate both the pars intermedia of the anterior lobe (PIAL) and paramedian lobule (PM) We have now studied systematically the termination in PM of the spinocerebellar climbing fibre paths ascending through the dorsal lateral and ventral funiculi of the cord The activity evoked in Purkinje cells by these paths was investigated in preparations with the cord transected in C3 except for one funiculus as described in previous papers concerned with the termination in the anterior lobe (see Larson Miller and Oscarsson 1969) Branching of climbing fibres to PIAL and PM was determined by antidromic activation as described by Armstrong *et al* (1971) The results are summarized in Fig 1 showing the projection zones in PIAL and PM Three zones A B and C are distinguished in PM Zone A consists of one part receiving information from the hindlimb (A_h) and one part receiving information from the forelimb (A_f) The climbing fibres terminating in zone A_h or A_f also send branches to one or both of the zones with the same designations in PIAL Fibres terminating in zones B and C of the PM send branches to corresponding zones in PIAL Each zone (or part of zone) receives climbing fibres which are activated by one or two spinal paths according to a complex pattern of

convergence presumably established at the olivary level (Fig 1 bottom) It is concluded that the same information reaches PIAL and PM through the climbing fibre paths and that the projection zones have a similar organization in these two areas except for the reversed somatotopy and the remarkable doubling of zone A in PIAL This zonal arrangement is supported by anatomical findings suggesting similar zones each with discrete efferent paths (Voogd 1969)

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C 15

Supernormality of Cerebellar Parallel Fibres The Effects of Changes in Potassium Concentration *in vitro*

By A R GARDNER MEDWIN, From *The Institute of Neurophysiology University of Oslo Norway*

In previous work it has been shown that the parallel fibres in the cerebellar cortex of the cat have a remarkable supernormal period after they have been activated (Gardner Medwin 1971) The conduction velocity of the fibres is increased by 15—20 % 20 ms after a conditioning stimulus and the stimulation threshold is lowered by up to 40 % The supernormal after effects are detectable for 100—200 ms after conditioning

A possible hypothesis to account for this supernormality is that it might be due to the accumulation of potassium in the confined interstitial spaces around the parallel fibres In the present experiments an *in vitro* preparation has been used to examine the effects on the conduction velocity and supernormality of varying the potassium concentration

Slices about 1/2 mm thick of the surface of the cerebellar cortex of guinea pigs were held submerged in a continuously flowing oxygenated saline solution kept at 38° C The potassium concentration in the fluid was initially 2 5 or 3mM (approximately that of cerebrospinal fluid and was varied over the range 0—7 mM *Compensating adjustments were made in the sodium concentration in order to keep* the tonicity constant In all other respects the saline composition was the same as that used by Richards and Sercombe (1970)

The responses observed in the slices differed from those in intact cats in two clear ways They lacked the second slow negative wave which is probably attributable to postsynaptic activity (Eccles Llinas and Sasaki 1966) and the stimulation threshold of the parallel fibres was raised rather than lowered following a conditioning stimulus The conduction velocity was nevertheless supernormal after conditioning by about 10 % with a 20 ms interval

The supernormality of the conduction velocity fell gradually and reversibly as the potassium concentration was increased At 5—7 mM potassium concentration there was no supernormality and the conduction velocities of the first and the second responses were the same Thus the supernormality of the conduction velocity was clearly sensitive to the extracellular potassium concentration The conduction velocity of single responses however, never increased by more than 5 % as the potassium concentration was increased This was less than the increase during the supernormal period at the low concentrations Thus the results do not fully support the hypothesis that changes in extracellular potassium could be responsible for the supernormality

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C 16

Antidromic Activation of Renshaw Cells and the Distribution of their Axonal Branches

By E Jankowska and D O Smith *Department of Physiology University of Goteborg Sweden*

Renshaw cells have been found to inhibit three different groups of neurones in the spinal cord motoneurones (Renshaw 1941, Eccles Fatt and Koketsu 1954) interneurones which mediate Ia reciprocal inhibition of motoneurones (Hultborn Jankowska and Lindstrom 1971 a) and other Renshaw cells (Ryall 1970 All these neurones and particularly Ia interneurones and Renshaw cells may be inhibited following stimulation of motor axons from the same as well as from neighbouring segments indicating that Renshaw cells have relatively long axons Hultborn *et al* 1971 a Ryall Piercey and Polosa 1971) This conclusion has now been confirmed and expanded by the results of an analysis of the antidromic activation of Renshaw cells

Action potentials of single Renshaw cells located in SI were recorded extracellularly with one microelectrode while their axonal branches were activated by weak current pulses passed through a second microelectrode within L7 and L6 (*cf* Jankowska and Roberts 1971) The lowest thresholds for activation of these branches (below 1 μA) and the refractory period of Renshaw cell soma 0 6—1 0 ms) and axons (0 75—1 25 ms) were within the same ranges as those for the previously studied Ia interneurones (Jankowska and Roberts, in press) Th antidromic impulses evoked only single spikes in the somas, under the same conditions orthodromic volleys generated a train of spikes

Renshaw cell axons were found to project for distances up to 13 mm (cf Hultborn et al 1971 a Ryall et al 1971) with more than half of those tested being activated from more than 2 mm rostral to their somas These were clearly longer than the distances from which the cells could be excited synaptically by stimulation of motor axons which in this study were found to be up to 1 8 mm (cf Ryall et al 1971) Axonal branching in motor nuclei was seen mainly within 1 mm in either the rostral or the caudal direction but in 3 cells it was found within 4 mm from their somas At longer distances the Renshaw cell axons could be activated either from an area ventromedial to the motor nuclei where Ia inhibitory interneurones and Renshaw cells are located or from the ventral funiculus where the stem axons of Renshaw cells apparently run Several cells were antidromically activated from all three locations Therefore it is likely that they terminated on motoneurones as well as on Ia interneurones and/or other Renshaw cells (cf Hultborn et al 1971 b)

The highest conduction velocities found for axons within the white matter (close to the somas) were nearly 30 m/s They decreased to 6—10 m/s at longer distances and/or for collaterals projecting towards the ventral horn The effects of Renshaw cells on their target cells located at some distance would thus be considerably delayed the conduction times from the Renshaw cell somas to the cells located at the same rostrocaudal level 2—3 mm rostrally and 10 mm rostrally are estimated to be about 0 1 msec 0 4—0 5 ms and above 1 0 ms respectively

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C 17

Electrogenic Na K pump in the Retinula Cells of Limulus Lateral Eye

By OLE P HANSEN *Zoofysiological laboratory B University of Copenhagen Denmark*

The presence of an electrogenic Na K pump has been shown in a number of preparations (Thomas 1969 Koike et al 1971) Recently Smith et al (1968 a b) put forth the hypothesis that the receptor potential in the ventral eye of Limulus is produced by a light mediated inhibition of a hypothetical electrogenic Na pump This hypothesis contradicts the widely held view that the receptor potential is generated by a light regulated conductance increase of the membrane (Fuortes 1959 Millechia and Mauro 1969)

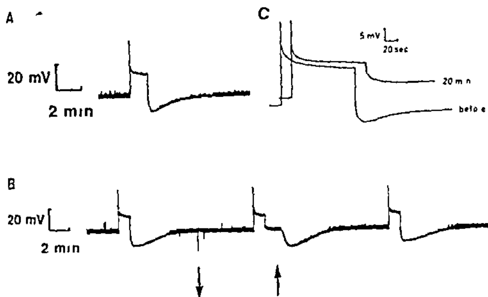


Fig 1 Pen recordings of the intracellular receptor potential of Limulus lateral eye retinular cells obtained in normal sea water (A) in K free sea water (B) and in 0 5 mM ouabain (C) In B the normal sea water has been substituted by K free sea water in the time interval indicated by the arrows The two recordings in C have been obtained before and 20 min after the start of application of 0 5 mM ouabain

The potential of the retinula cell has been measured with a conventional micro electrode technique By prolonged and intense illumination the depolarising receptor potential is followed by a pronounced transient hyperpolarization (Fig 1 A) similar in time course to the posttetanic hyperpolarization in nerves (Thomas 1969)

Fig 1 B shows the effect of K free sea water In the time interval indicated by the arrows the normal sea water was substituted by K free sea water Comparison of the light response in K free sea water with the responses in normal sea water shows that the receptor potential proper is unaffected by absence of K while the afterhyperpolarization is abolished Readmission of normal sea water triggers a hyperpolarization with similar time course and amplitude as the normal afterhyper polarization

Fig 1 C shows the receptor potential produced by illumination before and 20 min after application of 5 10^{-4} M ouabain There is little change in the amplitud of the resting and receptor potential The afterhyperpolarization however is com pletely abolished

These effects of K free sea water and ouabain strongly suggest that the afterhyper polarization is produced by the increased activity of an electrogenic Na K pump In contrast however to the hypothesis of Smith *et al* ouabain and K free sea water have no significant immediate effect on the resting and receptor potential

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C 18

Effect of Ouabain and Hypoxia on the Electrogenic Properties of the Isolated Frog (*Rana temporaria*) Gastric Mucosa

By G Flemström and K J Öbrink, *From the Institute of Physiology and Medical Biophysics, Biomedical Center, University of Uppsala, Sweden*

It has been shown that sodium ions are actively transported from the secretory (luminal) to the nutrient side of the isolated frog gastric mucosa (Flemström and Öbrink 1970) but only when the mucosa is in a hypoxic condition (Flemström 1971) An active transepithelial transport of sodium takes place also in mucosae isolated from several mammalian species but these preparations have a low rate of hydrogen ion secretion and a large production of lactate (*cf* Kitahara Fox and Hogben 1969) which may indicate that the preparations used have been hypoxic Since it is generally accepted that ouabain is a specific inhibitor of the outward sodium and inward potassium transport in cell membranes (Skou 1965) it was thought of interest to study the effects of ouabain (10^{-7} M) on the sufficiently oxygenated (pO_2 700 mm Hg in the bathing solutions) and the hypoxic (pO_2 300 mm Hg) frog gastric mucosa

It was found that ouabain inhibited the active net charge transport measured as short circuit current in the hypoxic mucosae In the fully oxygenated mucosae no such effect was obtained which corroborates the earlier finding that there is no active transport of sodium under this condition The inhibitory effect was obtained only when ouabain was given on the nutrient side which gives support to the earlier suggestion (Flemstrom 1971) that the mechanism for active electrogenic transport of sodium ions in hypoxic mucosae is assymetrically distributed and located on the nutrient side of the cells

Higher concentrations of ouabain are known to exert an unspecific toxic effect on the gastric mucosa (Davenport 1962) but in the experiments made here ouabain (10^{-7} M) caused no reduction of the hydrogen ion secretion rate which makes unspecific effects less probable

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C 19

The Importance of Blood Borne Factors for the Maintenance of the Liver Cell Membrane Potential

By M Folke, *Institute of Medical Physiology Dept 4, University of Copenhagen Denmark*

The electric potential difference (inside negative) across the cell membranes of rat liver *in vivo* is 40—50 mV (Williams *et al* 1971) This cell membrane potential (CMP) is 26—33 mV in isolated rat liver, perfused at 37 C with an oxygenated medium containing red cells (Folke 1971)

The cause of the lowered CMP *in vitro* is not clear One explanation namely irreversible damage to the liver during the procedure of isolation can be excluded on the following grounds

After 1—3 h of *in vitro* perfusion with rat blood the liver in 4 expts was connected to the vascular system of an anesthetized donor rat An increase in CMP from 26—31 mV to 41—52 mV occurred within 3—5 min The new level was maintained until the experiment was finished about 1 h later

In 3 other expts the blood in the isolated system was partially exchanged with freshly drawn rat blood This procedure also increased CMP to 40—45 mV but within 10—20 min after the exchange CMP had fallen again to approximately 30 mV

These observations suggest that maintenance of the normal liver CMP depends on factors present in fresh blood The factor(s) seem to be consumed by the liver but continuously resupplied in the living animal

Hormones would fit into such a picture Glucagon nor adrenaline and cyclic AMP are known to increase the hepatic CMP *in vitro* (Haylett and Jenkinson 1969 Friedmann *et al* 1971)

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C 20

Thyroid Hormone Secretion Induced by Sympathetic Stimulation

By A Melander E Nilsson and F Sundler *Departments of Pharmacology and Histology University of Lund Sweden*

Recent investigations have shown that under basal conditions *i e* when TSH secretion is eliminated exogenous catecholamines stimulate the secretion of thyroid hormone by a direct action on the thyroid follicle cells (Melander 1970) T

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C 18

Effect of Ouabain and Hypoxia on the Electrogenic Properties of the Isolated Frog (*Rana temporaria*) Gastric Mucosa

By G FLEMSTROM and K J ÖBRINK, *From the Institute of Physiology and Medical Biophysics, Biomedical Center, University of Uppsala, Sweden*

It has been shown that sodium ions are actively transported from the secretory (luminal) to the nutrient side of the isolated frog gastric mucosa (Flemstrom and Öbrink 1970) but only when the mucosa is in a hypoxic condition (Flemstrom 1971) An active transepithelial transport of sodium takes place also in mucosae isolated from several mammalian species but these preparations have a low rate of hydrogen ion secretion and a large production of lactate (*cf* Kitahara Fox and Hogben 1969) which may indicate that the preparations used have been hypoxic Since it is generally accepted that ouabain is a specific inhibitor of the outward sodium and inward potassium transport in cell membranes (Skou 1965), it was thought of interest to study the effects of ouabain (10^{-7} M) on the sufficiently oxygenated (pO_2 700 mm Hg in the bathing solutions) and the hypoxic (pO_2 300 mm Hg) frog gastric mucosa

It was found that ouabain inhibited the active net charge transport measured as short-circuit current in the hypoxic mucosae In the fully oxygenated mucosae no such effect was obtained which corroborates the earlier finding that there is no active transport of sodium under this condition The inhibitory effect was obtained only when ouabain was given on the nutrient side which gives support to the earlier suggestion (Flemstrom 1971) that the mechanism for active, electrogenic transport of sodium ions in hypoxic mucosae is assymetrically distributed and located on the nutrient side of the cells

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The marked difference between the K_m for "specific" ^{125}I insulin binding and the concentration of insulin which causes half of the maximal lipogenesis from glucose (7 μU/ml = 4 9×10^{-11} M) suggests that the majority of the receptors are 'spare receptors'

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C 22

Glucose Tolerance after Exercise in Juvenile Diabetics

By E D R PRUETT and S MÆHLUM, *Institute of Work Physiology, Oslo Norway*

It has long been known that exercise decreases the need for insulin in diabetics Furthermore, investigations have shown that tolerance for infused glucose is increased after exercise in non-diabetic subjects, and that this increase is dependent upon the severity and duration of the exercise work load (Pruett and Oseid 1970) In order to study the effect of exercise of different severities on glucose disappearance time in young male juvenile onset diabetics, four subjects were exercised at 20 %, 50 %, 70 % and 90 % of their maximal oxygen uptake (max VO_2) on a bicycle ergometer All subjects had been insulin treated for several years they were in the post absorptive state and without insulin on the day of the experiment The results of an intravenous glucose tolerance test (0 5 g/kg b w) given 15 min after the conclusion of the exercise were compared with a similar test given at rest It was found that, as in non diabetic subjects exercise for three hrs at 20 % of the subjects max VO_2 resulted in no change in glucose tolerance in any of the subjects Exercise at 50 % max VO_2 and higher on the other hand usually, induced an increase in glucose tolerance Fig 1 shows the change in tolerance for infused glucose due to 4 exercise work loads in one well regulated diabetic subject The disappearance curves after work at 50 % and 70 % max VO_2 in this subject are in good agreement with similar curves in non diabetic young men (Pruett 1971) The slope of the disappearance curves (logarithmic) for glucose after 50 % 70 % and 90 % max VO_2 are typical for non diabetic subjects after the same exercise In addition the disappearance curve for glucose infused 1 h after a 1 h exercise bout at 50 % max VO_2 was the same as that for glucose infused only 15 min after exactly the same exercise, but significally different from that obtained at rest in the same subject Thus it is seen that moderate to severe exercise can induce an effect upon glucose metabolism in diabetic subjects and that this effect is both potent and long lasting

findings raised the possibility of a direct influence of the sympathetic nervous system on thyroid hormone secretion, and this possibility was investigated in ^{131}I pretreated cats and mice, in which TSH secretion had been blocked by exogenous thyroxine A combination of light microscopic, fluorescence microscopic and bio-assay procedures was used

In both species electrical stimulation of the right cervical sympathetic trunk for 5—60 s induced a formation of intracellular colloid droplets in the thyroid, and this was accompanied by an increase in the blood ^{131}I levels The droplet formation was restricted to the thyroid regions that were supplied by this nerve, as judged from the distribution of catecholamine containing nerve terminals in thyroids from animals with a left side sympathectomy Noradrenaline intravenously induced a symmetrical formation of colloid droplets and an increase in blood ^{131}I Preatreatment with an alpha adrenergic blocking agent, phentolamine inhibited all these effects *Neither uni nor bilateral occlusion of the carotid blood flow for 60 s was* followed by any colloid droplet formation or any blood ^{131}I increase

The results suggest that sympathetic stimulation induces secretion of thyroid hormone, through a direct action on alpha adrenergic receptors in the thyroid follicle cells by noradrenaline released from intrathyroidal sympathetic nerve terminals

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C 21

Binding of ^{125}I-Insulin to Isolated Fat Cells

By S GAMMELTOFT and J GLIEMANN *Institute of Medical Physiology C University of Copenhagen Denmark*

The binding of ^{125}I insulin (0 1 mol of iodine/mol of insulin) to collagenase isolated rat fat cells was studied at 37° C using centrifugation of the cells through oil as a rapid and efficient method for removal of the incubation medium With 20 μU/ml of ^{125}I insulin 60—70 % of the label was displaced by the addition of unlabelled insulin 10^5 μU/ml The non displaceable (unspecific) insulin binding was proportional to the insulin concentration until at least 10^7 μU/ml The displaceable (specific) insulin binding exhibited Michaelis—Menten kinetics with a K_m of about 500 μU/ml (3 5 × 10^{-9} M) as determined in equilibrium binding experiments Assuming that ^{125}I insulin is almost fully biologically active (Freychet Roth and Neville 1971) it was calculated that each cell on the average possesses about 5 × 10^4 insulin receptors The dissociation rate constant (k_{-1}) was about 0 1 min^{-1} (varying between 0 08 and 0 17 min^{-1} in different experiments) and the association rate constant (k_1) was about 2 7 × 10 M^{-1}min^{-1} yielding a K_m of about 570 μU/ml (4 × 10^{-9} M)

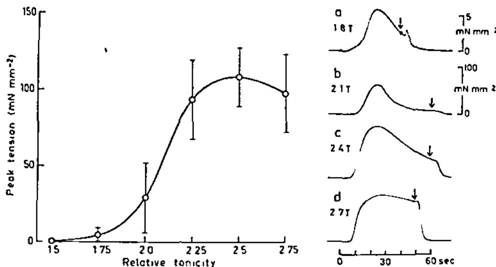


Fig 1 Left, relation between solution tonicity and peak tension of hypertonicity contracture
S mbol ... dard deviations indicated Curve
... fibre at four different tonicities
... r solution indicated by arrow
... applies to *c* and *d* as well

Twitch fibres were isolated from frogs semitendinosus muscles and mounted in a trough connected to fluid reservoirs by a stop cock system which allowed quick changes (1 s) of the bathing fluid Tetanus and contracture tensions were recorded isometrically at 2 2—2 3 μm sarcomere length Test solutions were prepared by addition of solid sucrose to Ringer solution to give an osmotic strength of 1 5—3 times normal (1 5—3 T)

Application of 1 75—3 T solution evoked a contracture which reached a maximum within 20—40 s *The largest peak tension was obtained with* 2 5 T (Fig 1 left), where the amplitude was 108 ± 13 mN mm⁻², about 40 % of the tetanic tension in Ringer solution (281 ± 25 mN mm⁻²) The contracture was phasic in 1 75—2 T and became more long lasting at higher tonicities (Fig 1 right) Contractures were reproducible up to 10 times if sufficient rest (30 min) was allowed between exposures to test solution

Tests with 2 5 T solution, performed 2 min after application of isotonic potassium methyl sulphate solution gave responses which reached 91 % (mean of 11 fibres) of the control tension Altered [Ca] 10 times below and 5 above normal (2 mM), had no effect on the tonicity—peak tension relation Addition of tetracaine (0 3 mM) suppressed 2 5 T contractures completely, in 0 1 mM peak tension was about 35 % of normal

The results suggest a direct effect of hypertonicity on the activator releasing mechanism

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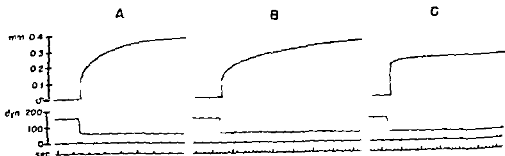
C 24

Contraction of Vascular Smooth Muscle Induced by Hyperosmolality

By Christer Andersson, Per Hellstrand, Borje Johansson and Anita Ringberg, *Department of Physiology and Biophysics, University of Lund, Sweden*

Smooth muscle of the rat portal vein shows a sustained increase of tension when exposed to a medium of twice the normal osmolality, produced by addition of sucrose or NaCl to the normal bathing medium The question arose as to whether this response was due to active contraction or simply to passive shrinkage of the tissue

In experiments with quick release during the osmotic response and during a K^+-contracture of comparable isometric force, the muscle showed about the same extent of total shortening (Fig 1) However the osmotically stimulated muscle had a lower shortening velocity Comparison of the length-tension diagrams for the osmotically induced tension increase and the K contracture showed that the two modes of stimulation gave maximal responses at about the same length The tension in ... induced by hypertonicity did not appear at a temperature of 10° C At this ... the K contracture was only reduced by about 50 per cent Eliminating ... from the external medium slightly reduced the magnitude of the response to hyperosmolality but a significant tension increase could be recorded even after incubation for 2 5 h in an EGTA-buffered medium with a Ca -concentration below



Isotonic shortening under a small afterload following quick release Upper tracing amount of shortening Lower tracing tension *A* muscle activated by K high solution with $[Ca^{2+}]_o$ adjusted to 0 25 mM to give the same isometric tension as in *B* *B* muscle in nominally Ca^{2+}-free, hypertonic medium (540 mOsm) *C* muscle in nominally Ca^{2+} free isotonic medium, stretched passively to give the same force as in *A* and *B* Note that the total amount of shortening is about the same in *A* and *B* The rate of shortening after release is slower in *B* than in *A* In *C* the immediate shortening due to series—elastic recoil makes up the major portion of the total change in length.

10^{4} M The K^{+} contracture is rapidly abolished in Ca^{++} free med um Hypertonicity caused increase in tension both in Ca free ' normal Krebs solution and in C ree K high solution indicating that the mechanical resp nse is not d pendent on the level of membrane potential

The results suggest that the osmotically induce tension increase is an is process rather than a purely physical effect due t he osmotic b h iour of cell. However, the dependence on the external Ca c centration i entire dif t from that of the K activated muscle It seems clea th t hyper molality not induce contraction by causing influx of Ca^{++} over the c membrane but by releasing Ca^{++} from intracellular stores or by acting thro mech an m er unrelated to Ca^{++}

C 25

Rapid Axonal Transport in Vitro of Glycoproteins

By A Edstrom H Mattsson and E Walum *Department of Zoophys ogy University of Göteborg Sweden*

Intraaxonal transport of amino acid labelled proteins has been extensi ely stud ed Less attention has been paid to the distribution of sub tances synthesized from precursors other than amino acids Of considerable biolog c l import nce are glycoproteins and glycolipids which constitute material present n the plasma membrane or associated with it

In order to study the synthesis and transport of glycopr tein *in v itro* s tem from frog *R temporaria* has been used The preparation which c sts of the dorsal ganglia the sciatic nerve the m gastrocnemius w placed in incubat n chamber having three compartments (A B C i e pa ts were eq uated from each other with silicone grease barriers (Edstrom and Mattsson 1971 Radioactive isotope was added to the A compartment which made it possible to follow the migration of H^3 glucosamine H^3 fucose and S^{35} sulfate labelled material from the ganglia, along the sciatic nerve towards the gastrocnemius muscle

Labelled glycoproteins in the sciatic nerve originated from the dorsal spinal ganglia and moved antidromically at a rate of 60—90 mm per day at 18° The distribution in different subcellular fractions extractability effect of cycloheximide and transport kinetics did not differ very much between glucosamine and fucose incorporated transported material Cycloheximide blocked the synthesis of material for export more effectively than the synthesis of stationary glycoproteins in the ganglia The rapidly transported TCA insoluble radioactivity was 85 % partic ulate and mainly associated with structures sedimenting in the microsomal fraction About 10 % of the TCA insoluble radioactive material was extracted by $CHCl_3$ methanol (2 1 v/v) and might accordingly be glycolipids S^{35} sulfate labelled TCA insoluble components probably proteoglycans were also rapidly transported but can only represent a quantitatively minor part of transported glycoproteins in this system

One role for the rapid transport may *a priori* be to deliver glycoproteins to the surface membrane, which by other studies is known to have such material located within it or in its near vicinity (Spiro 1970)

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C 26

The Effect of Hemorrhage on the Hepatic Metabolism and Hemodynamics

By NIELS KRARUP *Institute of Physiology University of Arhus Denmark*

It has been shown that hemorrhage causes a decrease in the number of perfused sinusoids in the liver (Seneviratne 1950) It might therefore be suspected that the functional capacity of the liver would be reduced after hemorrhage

In 12 chloralose anesthetized cats the blood volume was reduced 30 % after a control period of 150 minutes The functional capacity of the liver was followed by measuring the splanchnic elimination rate of ethanol at concentrations at which the elimination capacity is saturated Portal venous and hepatic arterial blood floow were determined as well as the mean arterial portal and hepatic venous pressure Furthermore the oxygen glucose pyruvate and lactate concentrations in arterial and hepatic venous blood were measured

The splanchnic elimination of ethanol was unchanged after bleeding in 7 of the cats (group A) and increased (32 %) in 5 (group B) The ethanol elimination in the control period was significantly larger in group A (36 μmol/kg min) than in group B (25 μmol/kg min In group A the splanchnic oxygen consumption and the arterial glucoseconcentration was unaltered after hemorrhage whereas both parameters increased in group B In both groups the arterial pyruvate and lactate concentrations as well as the splanchnic uptake of pyruvate increased after bleeding The splanchnic output of lactate and glucose did not change significantly

The changes in the hepatic hemodynamics were similar in the two groups Generally the total hepatic blood flow decreased (36 %) but to a smaller extent than the portal flow (65 %) indicating that the arterial fraction of the flow had increased (from 45 to 65 % The effect of hemorrhage on the hepatic arterial flow varied depending on the arterialblood pressure The hepatic arterial resistance decreased (35 %) after hemorrhage in contrast to the mesenteric vascular and portal resistance which both increased (125 and 190 % resp)

In conclusion the observed increase in portal venous resistance following hemorrhage was not accompanied by a decrease in the elimination rate of ethanol and consequently the number of perfused sinusoids was probably unaltered In cats in which the elimination rate of ethanol in the control period was low hemorrhage induced an increase in the elimination rate accompanied by an increase in the splanchnic oxygen uptake and arterial glucose concentration

Reference

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C 27

The Effect of Simultaneous but Opposite Alterations in Pulse Amplitude and Frequency on Arterial Baroreceptor Activity

By Ed Kendrick Bengt Öberg and Goran Wennergren *Department of Physiology University of Goteborg Sweden*

Arterial baroreceptor activity increases despite unchanged mean arterial pressure when pulse amplitude or pulse frequency increases (*e g* Gero and Gerova 1967) Little is known however concerning the net effect on baroreceptor activity when these two variables are concomitantly changed in opposite directions as occurs *e g* in hemorrhage It has been suggested (Neil 1952) that the reduced pulse pressure and increased heart rate present in this situation would result in a decreased baro receptor activity and that therefore compensatory reflex adjustments may be elicited even before mean arterial pressure is reduced

To analyse this problem the isolated carotid sinus regions of a recipient cat were perfused from one carotid artery of a donor animal By adjusting the position of the recipient cat below or above the donor the mean pressure in the cross perfused sinus regions could be kept constant despite blood pressure variations in the donor cat Alterations of carotid baroreceptor activity recorded as reflex changes in blood pressure and heart rate in the recipient must therefore depend solely on changes of pulse rate and/or pulse amplitude in the donor The recipient was vagotomized to eliminate buffering influence from aortic and heart receptors

The donor animal was bled and the isolated sinus regions consequently exposed to an increased pulse frequency and a reduced pulse amplitude while the mean sinus pressure was maintained constant These altered pulsation characteristics produced a reflex increase of blood pressure and heart rate in the recipient indicat ing a decreased baroreceptor activity In contrast when pulse frequency decreased but pulse amplitude increased in the perfused sinus regions consequent to efferent vagal stimulation in the donor the baroreceptor activity increased to judge from the reflex fall in blood pressure and heart rate in the recipient These observations suggest that alterations in pulse amplitude are more important than changes in pulse frequency in determining the carotid baroreceptor activity when the two variables are changed in opposite directions in various physiological cardiovascular response patterns The findings thus support Neil s suggestion that baroreceptor activity may be reduced in hemorrhage even at unchanged mean arterial pressure

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C 28

Mechanisms in Maintenance of Cardiac Output during Coronary Insufficiency

By J Kjekshus, J Lekven and O D Mjøs, *Institute for Experimental Medical Research, University of Oslo, Ullevål Hospital, Oslo Norway*

Following acute reduction of coronary blood supply, maintenance of cardiac output implies several compensatory mechanisms as yet not clearly defined.

In experiments in open chest dogs we have studied the early functional and metabolic responses to stepwise reduction in coronary blood flow The left coronary artery was cannulated and perfused from a carotid artery Coronary flow was measured with an electromagnetic flowmeter on the shunt and perfusion pressure was measured distal to an adjustable clamp Aortic flow and left ventricular pressure were continuously recorded Arterial and coronary sinus blood were sampled for estimation of myocardial oxygen consumption and lactate uptake

Previous studies have related a rise in left ventricular end-diastolic pressure to myocardial ischemia (Scheuer and Brachfeld 1966 Hamosh and Cohn 1971), changes in ventricular volume, however, is a more sensitive index (Spotnitz *et al* 1966) Ventricular dimensional changes were accordingly continuously recorded by pairs of distance gauges implanted into the left ventricular wall (Lekven *et al* 1972) As coronary perfusion pressure was gradually reduced coronary flow was maintained to a coronary pressure of 60—80 mm Hg Following further reduction in coronary pressure myocardial oxygen extraction was increased from 70 % to a maximal level of 82 % Cardiac output remained unchanged during gradual constriction of the shunt until oxygen supply was reduced by more than 15 % When myocardial oxygen supply was reduced to 50 % of control stroke volume averaged 74 %, further reduction was followed by a precipitous fall in stroke volume, and stable hemodynamic conditions could no longer be maintained The increase in myocardial oxygen extraction during coronary constriction accounted for a maximum of 5 % of the oxygen requirements. Although a significant increase in myocardial lactate efflux was observed anaerobic metabolism was found to contribute with a maximum of 4 % of the energy demand during ischemia. Regulation of cardiac output may be explained on a purely geometrical basis relating increased force of contraction to increased diastolic volume Consequently ventricular dilatation represents the most important compensatory mechanism in the maintenance of cardiac output and the external ventricular performance during acute coronary insufficiency Although cardiac contractility is gradually depressed during reduction of coronary blood supply, the concomitant increase in ventricular end-diastolic volume almost completely compensates for a reduction in myocardial oxygen supply of 40—50 %

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